Nitrogen fixation, soil quality and restoration trajectories in agricultural matrices of lowland Canterbury, New Zealand

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Shanshan Li

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The aim of the present study was to investigate the relationships between nitrogen (N)-fixing plants, associated symbiotic bacteria and soil properties, and to evaluate the ecological role of native N-fixing plants in the context of ecological restoration in agriculture landscapes of New Zealand. The work had a particular focus on a restoration project associated with a plantation forest to farmland conversion at Eyrewell in Canterbury. Approximately 150 ha has been set aside for ecological restoration, with an additional 150 ha of native plants being established on paddock and farm borders. The original dryland vegetation of nutrient-poor acidic soils is being restored and embedded within an intensively irrigated and fertilized agricultural matrix. A paucity of knowledge of the functional role of native N-fixing plants in New Zealand plant communities is probably surpassed by research addressing the widespread weed problem of invasive exotic N-fixing gorse and brooms.

Five endemic species of the Leguminosae (*Sophora prostrata*, *Sophora microphylla*, *Carmichaelia australis*) and Rhamnaceae (*Discaria toumatou*), and three exotic species of Leguminosae (*Securigera varia*, *Astragalus cicer* and *Cytisus proliferus*) were investigated. Laboratory experiments were carried out initially to isolate and identify N-fixing bacteria, with additional access to existing collections. N-fixing bacteria were then inoculated to native and exotic legumes in glasshouse experiments. Viable cultures of *Frankia* associated with *Discaria* were difficult to isolate and culture. A locally rare early-successional endemic species of non N-fixing plant (*Pomaderris amoena*, Rhamnaceae) was included in the study as a reference plant. Native and exotic legume species were grown with *P. amoena* in a pot experiment to investigate the relationship between their growth. Nitrogen, phosphorus and lime amendments were added to soils in a glasshouse pot experiment. A fertilizer trial was carried out in the field at Eyrewell. Native N-fixing species, associated assemblages of plants and soils in more natural plant communities in field in the Canterbury region were also located and described.

The results showed that inoculation of N-fixing bacteria on legumes improved plant growth and nodulation but this varied according to species and plants' age. Native N-fixing species were tolerant of but not responsive to high nitrogen agricultural soils. Urea fertilizer application led to increased soil acidity and phosphorus improved plant nodulation. There were some evidences that native species are adapted to New Zealand's acidic soils. Native N-fixing plants are able to improve the growth of other native plants, and maintain or increase available nitrogen in soil. This was quantified and the amounts were shown to be significant. Native N-fixers were found to naturally occur within plant communities that support a large number of other native species. Experimental research showed they may contribute a different and more beneficial role than exotic legumes in diverse native plant communities.

The findings of this research project indicated that N-fixing plants should be considered as an essential component of the restoration matrix in the ecologically-degraded landscapes of Canterbury and probably more widely in New Zealand. This research project has provided new insights into the interaction between N-fixing bacteria, N-fixing plants and soil properties, and the role of native N-fixers to restoration in agriculture landscapes.

Keywords: Nitrogen fixation, Rhizobium, Leguminosae, ecological restoration, agricultural landscapes, nitrogen, phosphorus, soil nutrients.

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Abbreviation of plant species and glossary

List of plant species in this study

Latin name	Abbreviation	Common name	Family
Astragalus cicer	*A. cicer*	cicer milkvetch	Leguminosae
Carmichaelia australis	*C. australis*	common broom	Leguminosae
Coronilla varia (*Syn. Securigera varia*)	*C. varia (Syn. S. varia)*	crown vetch	Leguminosae
Cytisus proliferus	*C. proliferus*	tree Lucerne	Leguminosae
Discaria toumatou	*D. toumatou*	wild Irishman	Rhamnaceae
Pomaderris amoena	*P. amoena*	tauhinu	Rhamnaceae
Sophora microphylla	*S. microphylla*	weeping kowhai	Leguminosae
Sophora prostrata	*S. prostrata*	prostrate kowhai	Leguminosae

List of chemical elements

Chemical elements	Abbreviation
Nitrogen	N
Phosphorus	P
Carbon	C
Ammonium	NH_4^+
Nitrate	NO_3^-

List of media

Abbreviation	Name of media
YMA	Yeast Mannitol Agar
YMB	Yeast Mannitol Broth
DPM	Defined Propionate Minimal Medium

List of molecular and biology glossary

Abbreviation	Description
DNA	Deoxyribonucleic acid
PCR	Polymerase chain reaction
RNA	Ribonucleic acid
16S rRNA	16S ribosomal RNA
RecA	DNA recombination and repair protein
NifH	Nitrogenase iron protein H
NodD	Nodulation protein D
RecA gene	A gene which encode RecA protein
NifH gene	A gene which encode NifH protein
Nod factors	Signaling molecules produced by bacteria during the initiation of nodules
ATP	Adenosine triphosphate (high-energy molecule that stores the energy)

Chapter 1

Introduction

1.1 General introduction

Although the losses of natural resources in New Zealand may not be as serious as in other industrialized countries, a number of topical and serious environmental concerns include atmospheric gas emissions, water pollution and loss of native habitats (Duncan, 2014; Kelsey, 2015; Ross, 2015; Simberloff et al., 2013). Over 160 plant species and 40 different species of birds were classified as threatened in 2000 in New Zealand (De Lange et al., 2013; Robertson et al., 2013). On the Canterbury plains, on South Island, much native vegetation was destroyed following settlement of both Maori and Europeans (Evison, 1993; McGlone, 1989; McWethy et al., 2010). Less than 10% of the Canterbury region was forested when Europeans arrived (Bauhus et al., 2010; McWethy et al., 2010). Today, the original vegetation is only present at restricted locations in the alpine zone and foothills, and on Banks Peninsula. Grasslands and arable farming predominate on the Canterbury plains (Godley, 1975; Scott, 1979). Nowadays, conservation and restoration are major issues that are widely discussed by researchers, government and the general public (Saunders & Norton, 2001). This is not just because of the loss of native vegetation, but is also due to the loss of associated environmental values of native habitats. New Zealand agriculture produces much wealth but also contributes to deterioration of landscapes through soil degradation, water contamination and loss of biodiversity (Glade, 2003).

Generally, New Zealand's environment is undergoing pressure from climate change in addition to an increased intensification of land usage for farming (Orwin et al., 2015; Schipper et al., 2014). Nitrogen leached into water bodies has increased dramatically since 1990, caused by dairy farming and nitrogen fertilizers (De Klein, 2001; McKergow et al., 2016; Pinxterhuis et al., 2015). Nutrient spillover, soil erosion and compaction are also contributed by intensive farming add a nutrient spillover relevance. Most soils in New Zealand originally supported forests with limited stores of crucial nutrients; nitrogen and phosphorus were removed faster than their replacement through natural processes. Fertility amendments are required to support pasture and crop production. Nitrogen, P, lime and other fertilizers are applied regularly to maintain productivity. Introduced clovers, lucerne, and lotus have also been planted extensively to fix nitrogen in pasture, as legumes are a cheaper way to enhance soil nitrogen than use of nitrogen fertilizer.

There is a relatively small number of native N-fixing species in New Zealand, none of which are appropriate or considered to be applicable to agricultural systems. Native N-fixing species only present at 1.6% of the New Zealand flora, compared to introduced N-fixing species which represent 7.5% of the flora in New Zealand. Four genera of native *Leguminosae* are present in New Zealand, with a total of 34 indigenous species. *Carmichaelia*, *Sophora*, *Clianthus* and *Montigena* include trees, shrubs and herbs (Tan, 2014; Weir et al., 2004). Different species of *Rhizobium* are known to be associated with these native legumes to form nodules and consequently to fix nitrogen. Actinomycetes also form nodules with some non-leguminous species, but are less prevalent (Gordon & Wheeler, 2012). Worldwide, a range of plant genera are symbiotic with actinomycetes such as *Alnus* (Betulaceae), *Casuararina* (Casuarinaceae) and *Purshia* (Rosaceae). Some *Coriaria* (Coriariaceae) species which fixs nitrogen are native to New Zealand (Silvester, 1968). *Discaria toumatou* (Rhamnaceae) is the only *Discaria* species native to New Zealand, that is known to fix nitrogen with actinomycetes (Torrey, 1978).

1.2 Aims and objectives

1.2.1 Aims of the research

The aims of this project are to:

(i) investigate the relationship between N-fixing plants, associated symbiotic bacteria and soil properties, and

(ii) evaluate the ecological role of native N-fixing plants in the context of ecological restoration in agricultural landscapes.

The objectives of the experimental work are to:

1) isolate N-fixing related bacteria from exotic legumes (*Coronilla varia* (*syn. Securigera varia*), *Astragalus cice*r and *Cytisus proliferus*) with identification (Chapter 3).

2) investigate the influence of N-fixing bacteria on growth of native and exotic legumes, and interactions with the growth of *Pomaderris amoena* (Chapter 4).

3) investigate the growth response of native N-fixing species (*Sophora microphylla*, *Sophora prostrata*, *Carmichaelia australis* and *Discaria toumotou*) and growth comparison between these N-fixing plants and a non N-fixing plant (*Pomaderris amoena*) to different soil nutrient conditions in a glasshouse conditions (Chapter 5).

4) evaluate the interaction between growth of native N-fixers and fertilizer applications (nitrogen and phosphorus) in agricultural soils in field conditions (Chapter 6).

5) explore the community associations and soils at locations with the native N-fixers (*Sophora microphylla*, *Sophora prostrata*, *Carmichaelia australis* and *Discaria toumotou*) that are naturally found in Canterbury (Chapter 7).

Aims and objectives are based on the following questions:

1) Do N-fixing plants have a role on soil restoration in agriculture landscapes?
2) What effects of native N-fixing plants with their symbiotic bacteria on different nutritional agriculture soils?

1.2.2 Thesis structure

This thesis is structured with a literature review describing the research background (Chapter 2), five experimental chapters (Chapter 3 to 7) and a final discussion and conclusions (Chapter 8). Chapter 3 focused on microbiology. Chapter 3 and 4 described laboratory and glasshouse pot experiments. Chapter 5 and 6 reported a field trial and a field survey respectively.

Chapter 2

Background and literature review

2.1 History of New Zealand agriculture

Agriculture is the most important economic driver in New Zealand. The large-scale conversion of land to agriculture has occurred for only about 200 years (McGlone, 1989), since the settlement of Europeans (Norton & Miller, 2000). Over 30% of the conversion happened from 1900 until the 1970s, alongside an increasing amount of introduced vegetation (Molloy, 1980). Vegetation destroyed by fire led to the losses of native grasslands with the increasing of grazing during the last century in both the lowlands and high country; top-dressing, over-sowing and intensification of agriculture improved by new technology has progressively taken place throughout the North and South Island, and especially in the South Island plains (Molloy, 1980).

2.2 Restoration in agriculture landscapes

The present study is concerned with ecological restoration in the intensive agricultural landscapes of New Zealand. Understanding the principles of ecological restoration is clearly important to ecosystem recovery following disturbance (Jackson & Hobbs, 2009). Although general theories of ecological restoration are well known, they are rarely implemented in practice in large-scale landscapes (Holl et al., 2003). The gap between theory and field experiment is one of the current challenges of ecology. Agriculture land use, including farming and grazing, has obviously created major disturbance and has led to ecological habitat loss and land degradation (Swinton et al., 2007). However, it is now recognized that ecological components of agricultural landscapes play an important role in delivering ecosystem services. Restoration of natural areas within agriculture landscapes may benefit both the agricultural industry and the natural environment. The agriculture landscape is, of course, part of a larger cultural landscape. Moreira et al. (2006) considered that cultural landscape restoration has some differences to ecosystem restoration; whereas ecosystem restoration always concentrates on a single ecosystem, cultural landscape restoration focuses on both land use and ecosystem.

2.3 The role of nitrogen-fixers in succession

Plants assemblages change during ecological succession and many species occur in just one phase of the succession, either in pioneer or mature phases or somewhere in between (Walker & Del Moral, 2003). Some plant and soil trends are universal during succession, such as an increase in plant biomass and soil nutrients (West et al., 2012). A common sequence of succession follows a trajectory of mosses, small herbaceous and lichens then N-fixing shrubs (Walker et al., 2007). In primary succession, no or little nitrogen is available from the substrate and only nitrogen-fixing species are able to establish, improve soil nitrogen and also facilitate the growth of other plants that concurrently or subsequently establish (Vitousek & Walker, 1989). Over time, the soil nitrogen content is increased by largely dead plants, leaves and roots mixing into the soil (Stevenson & Cole, 1999), allowing other plants to use this source of nitrogen to grow. In New Zealand, native nitrogen-fixing plants such as blue-green algae, some lichens, *Sophora* species, *Discaria toumatou* and some native brooms are all appeared in early primary succession (Bellingham et al., 2005; McQueen et al., 2006). Since Europeans arrived, introduced N-fixing plants such as *Trifolium* species, *Lupinus arboreus*, *Cytisus scoparius* and *Ulex europaeus* are frequently also present and may dominate early successions (Bellingham et al., 2001).

2.4 Biological and symbiotic nitrogen fixation

Through nitrogen fixation, molecular nitrogen is reduced to ammonium which is used in biological systems for organic compound synthesis (Cheng, 2008). The nitrogen-fixing reaction is catalyzed by nitrogenase which can be represented by the formula:

$N_2 + 8H^+ + 8e^- + 16ATP \rightarrow 2NH_3 + H_2 + 16ADP + 16Pi$ (Halbleib & Ludden, 2000).

ATP, as metabolic energy, is used for the reduction of dinitrogen to ammonia in this reaction. There are two modes of biological nitrogen fixation in natural ecosystems, from plant-associated and free-living bacteria (Figure 2.1) (Herridge et al., 2008).

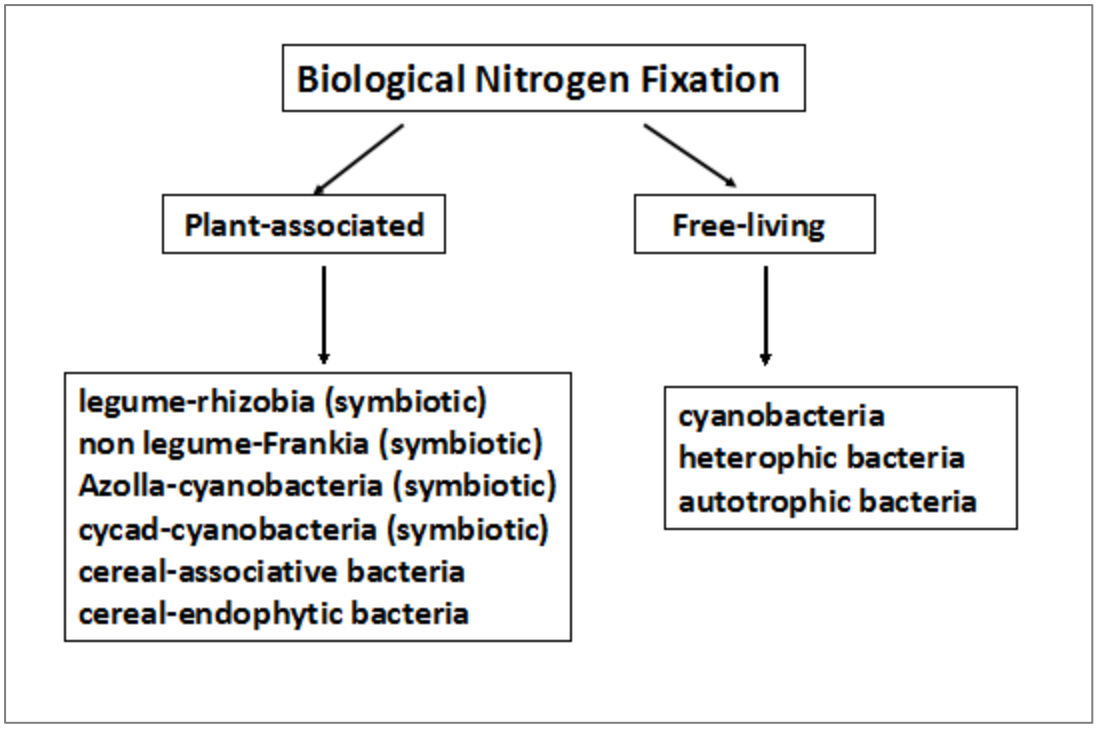

Figure 2.1 Biological nitrogen fixation pathways in natural ecosystems (Herridge et al., 2008).

As above, there are six forms of plant-associated biological nitrogen fixation, four of which are symbiotic. The legume-rhizobia symbiosis is most commonly studied in the context of agriculture and ecological restoration. In symbiotic nitrogen fixation, a tight relationship is established between prokaryotic organisms and their host plants which provide an appropriate rhizosphere environment (Mylona et al., 1995).

Apart from the symbiotic plant-associated model, associative and endophytic bacteria with cereals and grasses are also included in the plant-associated biological nitrogen fixation system. The difference between symbiotic is the N-fixing bacteria of non-symbiotic either exist in rhizosphere soils which associate with plant roots or else in healthy plant tissues (Boddey & Dobereiner, 1995; Elmerich & Newton, 2007). Different species of associated bacteria have improven to be able to fix nitrogen, such as species of *Achromobacter*, *Acetobacter*, *Azototacter*, *Bacillus*, *Clostridium*, *Corynebacterium*, *Desulfovibrio*, *Enterobacter*, *Herbaspirillum*, *Lignobacter*, *Mycobacterium* and *Rhodospirillum*, providing a nitrogen source to cereal plants and grasses (Wani, 1990). Species of *Klebsiella*, *Gluconacetobacter*, *Herbaspirillum* and *Azoarcus* have been reported to fix nitrogen as endophytic bacteria which isolated from non N-fixing plants including rice and other cereal plants (Elmerich & Newton, 2007). However, associative and endophytic bacteria are outside the scope of the present study.

2.5 Biological nitrogen fixation and agriculture

Legumes represent a limited range of plants that are able to fix nitrogen. This special ability has been utilized for a long time by agriculturists, for increasing soil fertility by periodically planting legumes on cultivated ground for crops, pastures and fodders in agricultural systems (Herridge et al., 2008). Legumes have always been routinely used in crop rotations to produce high-protein forage for livestock, additionally, to benefit subsequent crops through nitrogen supplied by legumes.

Legume-rhizobia system fixes more nitrogen than other plant-associated systems (Herridge et al., 2008). The forage legumes, such as alfalfa, clover and sweet clover usually give more nitrogen to subsequent crops than grain legumes like soybean and common bean (Hirsch et al., 2001). Worldwide, crop legumes are present on 186 Mha of land and fix 115kg nitrogen ha^{-1} $year^{-1}$ in agricultural systems. In comparison, 110 Mha of land in pasture and fodder legumes fixes 110-227 kg nitrogen ha^{-1} $year^{-1}$ (Smil, 1999). Peoples et al. (2009) reported that, compared with the global estimates of annual nitrogen fixation (20-22 Mt) and the amount of nitrogen removed in grain each year (17 Mt), the residual fixed nitrogen that is contributed into agricultural soils is only small. Some reports have shown crops yields are often greater with legumes than with fertilizers alone (Chalk, 1998; Kirkegaard et al., 2008). Crops yields especially depend on the amount of biological nitrogen fixation from legumes and the amount which removed by harvesting of legumes. The net addition is considerable for forage legumes while grain legumes remove more nitrogen which is accompanied with harvesting than is added by fixation (Anglade et al., 2015; Reynolds et al., 1994).

Biological nitrogen fixation was the dominant source of reactive nitrogen input to agriculture prior to the industrial revolution. As more agriculture production has been required, reactive N has substantially increased through the use of nitrogen fertilizers (Canfield et al., 2010). Intensive grazing systems require a high soil nitrogen but also increase the risk of nitrate leaching (Decruyenaere et al., 2007). It has been argued that the efficiency of fertilizer nitrogen and industrial nitrogen fixation must be suitable for long-term sustainability of the planet, not just for the short-term of increasing agricultural production, and should take account of associated problems of gaseous losses which contribute to global warming, leaching and degradation of water (Marschner & Rengel, 2007; Vitousek et al., 1997). This might require agricultural systems to return to being more reliant on biological nitrogen fixation and less on “human-made” nitrogen in the future (Herridge et al., 2008).

2.6 Legume and rhizobia symbioses

Legume-rhizobia symbioses are the most common partnership for nitrogen fixation in nature, partly because legumes are widely represented in the terrestrial ecosystem. Furthermore, rhizobia are fast growing and are easy to culture in the laboratory (Verghese & Misra, 2002). Around 90% of the nitrogen which is used by legumes comes from nitrogen fixation (Franche et al., 2009). Leguminous plants are therefore of considerable importance in both agricultural and infertile marginal land (Phillips, 1980). Rhizobia are gram-negative bacteria and only infect leguminous plants; almost 90% of legumes are able to establish a symbiotic system with these bacteria that are commonly referred to as "rhizobia" (Jorgensen & Fath, 2008; Verghese & Misra, 2002).

Currently, there are 113 described species of bacteria that are able to form nodules with legumes. They are classified into two groups named as alpha-proteobacteria and beta-proteobacteria (Willems, 2006). Alpha-proteobacteria is recognized to contain 11 genera including *Aminobacter*, *Azorhizobium*, *Bradyrhizobium*, *Devosia*, *Ensifer*, *Mesorhizobium*, *Methylobacterium*, *Microvirga*, *Ochrobactrum*, *Phyllobacterium* and *Rhizobium*. Beta-proteobacteria contains 3 genera are *Burkholderia*, *Cupriavidus* and *Herbaspirillum* (Laranjo et al., 2014; Moulin et al., 2001; Relman et al., 1992; Tan, 2014). Some rhizobia species are limited to nodulation of specific legume species while others nodulate a range of legume hosts within subfamilies (Dénarié et al., 1992; Hirsch et al., 2001). This situation is due to the complex chemical signaling of infection between bacteria and plants (Dénarié et al., 1992). *Mesorhizobium* species and *Rhizobium* species have been identified to form nodules with New Zealand native legumes whereas *Ensifer* and *Bradyrizbobium* were isolated from introduced weed legume species in New Zealand (Andrews et al., 2015; Tan, 2014; Weir et al., 2004). Some examples of rhizobial genera and their host plants shown in Table 2.1.

Table 2.1 Rhizobial genera and strains known to nodulate legume crop plants in New Zealand soils (from Andrews et al., 2015).

Legume	Rhizobia	Reference
Clovers (*Trifolium* spp.)	*Rhizobium leguminosarum* bv. trifolii	ICMP recommended inoculum; Tan 2015
Lucerne (*Medicago sativa*)	*Ensifer meliloti* *Rhizobium*	ICMP recommended inoculum; Liu 2014; Wigley *et al.* 2015
Lotus pedunculatus	*Bradyrhizobium* ICMP 5798, *Bradyrhizobium* ICMP 5942 *Bradyrhizobium*	ICMP recommended inocula Liu 2014
Lotus corniculatus	*Mesorhizobium loti*	ICMP recommended inoculum
Lupins (*Lupinus* spp.)	*Bradyrhizobium* ICMP 8377 *Bradyrhizobium*	ICMP recommended inoculum BASF recommended inoculum
Lupinus polyphyllus	*Bradyrhizobium*	Ryan-Salter *et al.* 2014
Field pea (*Pisum sativum*)	*Rhizobium leguminosarum* bv. viciae	ICMP recommended inoculum
Soybean (*Glycine max*)	*Bradyrhizobium japonicum*	ICMP recommended inoculum

In legume-rhizobia systems, bacteria enter an epithelial cell of the root then migrate into cortex, within an intracellular channel that grows through one cortex cell after another constructed by root cells (Oldroyd et al., 2009; Udvardi & Poole, 2013). This kind of process is referred to as an “infection thread” which is formed only in response to infection (Somasegaran & Hoben, 2012). Then, rhizobia fill the cell to form nodules for nitrogen fixation (Somasegaran & Hoben, 2012; Stacey et al., 2006). In the process of nodulation and nitrogen fixation (Figure 2.2) a complex molecular dialogue takes place, involving Nod factors synthesized by the bacterium and flavonoids released by legume roots, so that both symbiotic partners can recognize each other and initiate nodulation (Laranjo et al., 2014; Oldroyd, 2013; Remigi et al., 2016; Stacey et al., 2006). While the development of nodules dependent on rhizobia, it is a well-coordinated developmental process of plants. As a safe place for a rhizobium, legumes provide nutrients to the rhizobium which they synthesize the large amounts of ATP to convert nitrogen to ammonia (Day et al., 2001; Kiers et al., 2003). Legumes can supply nitrogenase (a key enzyme which encodes by *nif* genes) for the process of nitrogen fixation (Remigi et al., 2016).

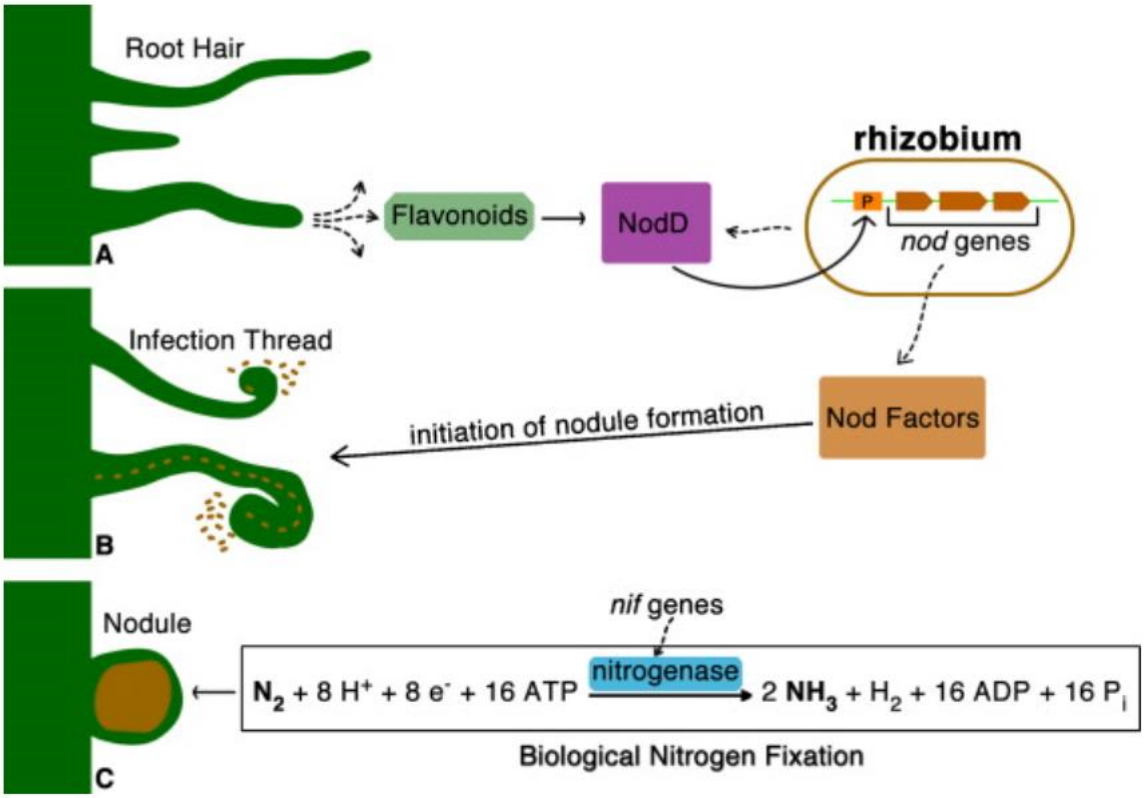

Figure 2.2 Schematic overview of the nodulation process and biological nitrogen fixation (from Laranjo et al., 2014). Interpretation of the molecular glossary is shown in previous "Abbreviation of plant species and glossary" section.

2.7 New Zealand nitrogen-fixing plants

2.7.1 New Zealand Leguminosae

Native Leguminosae

Four different genera of native Leguminosae exist in New Zealand (Table 2.2) contributing a total of 34 species: 23 *Carmichaelia* (broom) spp., 8 *Sophora* (kowhai) spp., 2 *Clianthus* (kakabeak) spp. and 1 native *Montigena* (scree pea) spp. (Heenen, 1998). *Carmichaelia* and *Sophora* (Figure 2.3) have large numbers of species compared to the other two genera and are widely distributed throughout New Zealand.

Table 2.2 Native genera of Leguminosae in New Zealand (from Tan, 2014).

Family	Fabaceae (Leguminosae)			
Sub family	Papilionoideae (Faboideae)			
Tribe	Galegeae			Sophoreae
Subtribe	Carmichaelinae			–
Genus	*Carmichaelia*	*Clianthus*	*Montigena*	*Sophora*
Species	*C. appressa*	*C. puniceus*	*M. novae-zelandiae*	*S. chathamica*
	C. arborea	*C. maximus*		*S. fulvida*
	C. astonii			*S. godleyi*
	C. australis			*S. longicarinata*
	C. carmichaeliae			*S. microphylla*
	C. compacta			*S. molloyi*
	C. corrugata			*S. prostrata*
	C. crassicaulis			*S. tetraptera*
	C. curta			
	C. glabrescens			
	C. hollowayi			
	C. juncea			
	C. kirkii			
	C. monroi			
	C. muritai			
	C. nana			
	C. odorata			
	C. petriei			
	C. stevensonii			
	C. torulosa			
	C. uniflora			
	C. vexillata			
	C. williamsii			

Figure 2.3 *Sophora microphylla* (left) and *Carmichaelia australis* (right) (from Google Image).

Carmichaelia has been reported to enhance foliar nitrogen of two common native woody plants, *Griselinia* and *Weinmannia*, and may facilitate future establishment of successional plants (Bellingham et al., 2001). *Carmichaelia* has 15 species endemic to the eastern South Island (Wagstaff et al., 1999), such as *Carmichaelia australis, Carmichaelia appressa,* and *Carmichaelia compacta* (Heenen, 1995, 1996). Many of New Zealand's native brooms grow in open or disturbed habitats and are adaptable to a range of different environments (Heenen, 2000; Mildenhall, 1980). There is a great proliferation of *Carmichaelia* spp. in the eastern South Island, probably due to the isolation created by the geological and soil variability, and the prevalence of dry, open habitats (O2 Landscapes, 2017). *C. glabrescens*, *C. torulosa*, *C. muritai*, *C. stevensonii*, and *C. carmichaeliae* are flowering species from Marlborough and eastern Canterbury. Together with *C. glabrescens* they have received some interest from gardeners but are seriously threatened in the wild (De Lange et al., 2009; Gruner, 2003; Heenen, 1995). Flower colours of *Carmichaelia* range from pale lavender through to intense pink. Northern areas of New Zealand are suitable for cultivation of two species (*C. australis* and *C. williamsii*) that naturally occur in the North Island (OL, 2017). *Carmichaelia williamsii* is a rare coastal species, with comparatively large, cream-yellow flowers, and a marked weeping habit (OL, 2017). *Carmichaelia australis* (included in the present study) is a native broom or shrub with branches up to 100 mm diameter (Heenen, 1996).

Sophora contains more than 50 species worldside, and has been found in both subtropical and milder regions. Eight endemic *Sophora* species exist in New Zealand, mainly as trees and shrubs, some with a prostrate habit (Thomas & Spurway, 2002). Only a few species of *Sophora* are naturally present in the South Island, including *S microphylla* and *S. prostrata* (Heenan et al., 2001). Some species are associated with special geologies, for example, *S. godleyi* is found over sandstone and mudstone, and *S. longicarinata* over limestone and marble near Nelson northwest in New Zealand (New Zealand Plant Conservation Network, 2014).

Different nitrogen-fixers have different morphologies, even from the same genera; for example, *S. microphylla* is a small-leaved native tree up to 25 m tall while *S. prostrata* is small-leaved shrub up to 2 m tall, both naturally found on South Island (Wardle, 1963, 1991). *S. microphylla* is present in isolated stands within alluvial forest (NZPCN, 2014). *S. microphylla* exist throughout New Zealand but infrequent in parts of Northland and it is currently under-represented within natural reserves (Heenan et al., 2001). Additionally, *S. microphylla* can be found along mainly riparian forest in the North Island and some habitats from coastal wetlands to inland scrub communities in the south of Hamilton (Mildenhall, 1980). *Sophora prostrata* is native to the area from Marlborough to southern Canterbury, and is easy to cultivate and thus a versatile plant for landscape gardening (Heenan et al.,

2001). *Sophora prostrata* has recently had increased appreciation by landscapers and garders due to the sculptural form and especially attractive orange and yellow colouration (NZPCN, 2014). It is well established for planting either in natural form or hedge. It is also could be put in pots for long-term cultivation due to the toleration of root restriction, sun exposure, wind and drought (Williams, 2006).

Exotic Leguminosae

New Zealand also contains 113 fully naturalized and 53 casual exotic nitrogen-fixing species (Thomas & Spurway, 2002), totally 166 exotic legumes compared to 34 native species. *Cytisus* spp. (brooms), *Ulex europaeus* (gorse) from Europe, and *Acacia* and *Albizia* (wattles) from Australia have all been naturalized in New Zealand since the 19th century (Cameron, 2000; Weir et al., 2004). The exotic legumes have higher seed production, seeds that typically disperse over a large area, more robust seedbanks, and often the plants can live for many years (Weir, 2006). These properties have meant they are widely dispersed throughout a wide range of landscapes in Canterbury.

Securigera varia, *Astragalus cicer*, and *Cytisus proliferus* (Figure 2.4) are the selected exotic species used in the present study, largely because it was considered there is scope for their use in restoration (R. Lucas pers. Com., 2015). *Securigera varia* is a low-growing legume native to Europe and Asia that has been used for erosion control in North America (Thompson et al., 2014). This species has been naturalized since 1906 in New Zealand, but there has been little earlier study of this species (NZPCN, 2010); it appears to have only has been found in the Lake Pearson area in the South Island (Terrain, 2017). *Cytisus proliferus* is a small evergreen N-fixing shrub that is well known as fertilizer plants, and it is suited to sandy and well-drained soils (Gutteridge, 1994). Snook (1986) reported that *Cytisus proliferus* could be used as a high production fodder crop for grazing in New Zealand. Douglas et al. (1996) considered that *Astragalus cicer* may be useful for revegetating dry lands in the North and South Islands, and as potential forage sources in these areas. This genus contains a large amount of species worldwide, a few of which are toxic to livestock (Rios & Waterman, 1997). *Astragalus cicer* naturally grows in areas in North America and Europe with low (350 mm) annual rainfall (Acharya et al., 2006; Nichols et al., 2016; Townsend, 1993). It establishes and thrives well in low-nutrient and disturbed soils in New Zealand (Davis, 1981; Nichols et al., 2016). It has been suggested that it would be beneficial to select more soft-seeded accessions than the genotype(s) that currently exist in New Zealand (Nichols et al., 2016).

Figure 2.4 *Securigera varia* (left), *Astragalus cicer* (middle) and *Cytisus proliferus* (right) (Imagery from Google Image).

2.7.2 New Zealand's native Rhamnaceae

Over 50 genera of plants belong to the Rhamnaceae family which includes trees, shrubs, climbers, and one herb (Richardson et al., 2000). *Discaria toumatou* (Figure 2.5) is a native species of the Rhamnaceae (Table 2.3) that is a known nitrogen-fixing plant in association with *Frankia* (Primack, 1979). *Discaria toumatou* is a small-leaved, spiny deciduous shrub which is common in the South Island high country normally up to 2-3 meters high, is also found with small pupolations in the North Island, and it has been changing with intensive farming (Duguid, 1976). The species is known to be associated with tussock, and only vulnerable to grazing when young because it develops spiny growth (McQueen et al., 2006). It also provides the locale for birds, and food (pollen and nectar) for insects (Keogh, 1990).

Pomaderris (Figure 2.5) is one genus of the Rhamnaceae that comprises about 70 species throughout Australia and New Zealand (Millott & McDougall, 2005). Eight indigenous species and one exotic have been found in New Zealand; four of the indigenous species are endemic (Table 2.3) (Breitwieser et al., 2010). There is no current evidence showed that *Pomaderris* are nodulated in New Zealand. *Pomaderris amoena* as one of the species commonly up to 1m tall with small narrow wrinkled leaves and white or yellow flowers, and it grows very well after establishment. It has been found in Whakatiwai Regional Park in North Island (Stanley, 2007). In South Island, it is generally scarce, reaching its southern limit at Eyrewell Forest in Canterbury (Dollery, 2017). This species was included in the present study due to its threatened status and rarity at the main study site at the

Eyrewell forest (a studying site in the present research, details in #6.2.1), and thus its importance as a component of the restoration effort at the site. No prior study of this species is known to exist.

Table 2.3 Species of Rhamnaceae in New Zealand (Breitwieser et al., 2010).

Family	Rhamnaceae	
Genus	Pomaderris	Discaria
Species	*Pomaderris amoena* (Endemic) *Pomaderris rugosa* (Endemic) *Pomaderris hamiltonii* (Endemic) *Pomaderris kumeraho* (Endemic) *Pomaderris apetala* (Non-endemic) *Pomaderris paniculosa* (Non-endemic) *Pomaderris phylicifolia* (Non-endemic) *Pomaderris prunifolia* (Non-endemic) *Pomaderris aspera* (Exotic)	*Discaia toumatou* (Endemic)

Note: Only *Discaria* is know to fix nitrogen.

Figure 2.5 *Pomaderris amoena* (left) and *Discaria toumatou* (right) (from Google Image).

2.8 The role of nutrients on nitrogen-fixing plants and soils

Environmental factors, such as temperature, water availability, soil pH and soil nutrient situations are known to limit the process of nitrogen fixation and the growth of legumes (Weisany et al., 2013). Of course macronutrients such as nitrogen, sulphur and phosphorus, and micronutrients like iron, manganese, copper and zinc are essential for the growth of all plants (Horst, 2011). Nitrogen-fixing species may have less requirement for nitrogen compared to non N-fixing plants. However, some mineral nutrients are required by legumes for nodule development and function (O'Hara, 2001); several mineral elements (P, S, K, Ca, Mg, Fe, Mn, Cu, Zn, Mo, B, Ni and Co) have been shown to be essential for symbiosis to occur (Arnon & Stout, 1939; Weisany et al., 2013). Nutrient deficiencies could negatively affect legume-rhizobia symbiosis at an early stage of nodule development (Horst, 2011).

Nitrogen is essential for plant growth due to the nitrogen assimilation into amino acids is important for building plant proteins (Masclaux-Daubresse et al., 2010). Soil does not always has a sufficient nutrients supply to plants, thus, N-fixing plants are advantageous than other plants. However, conversely, an excess soil nutrients may not stimulate the growth of plants (Van-Wijk et al., 2003). There is a diversity of forms of nitrogen in the soil, as either inorganic or organic nitrogen compounds, and plants are able to use different forms of nitrogen to various degrees (Näsholm et al., 2009). Nitrogen-fixing plants are able to obtain organic-derived nitrogen from soil as well as inorganic nitrogen obtained from symbiotic fixation or decomposition of organic matter. Normally, addition of nitrogen fertilizers is not recommended for legumes, although, a small application of nitrogen fertilizer may be optimal for young plant growth before nodules are fully developed (Hardarson et al., 1984). Larger amounts of nitrogen fertilizer may have adverse effects on yield but this is not always the case. Hardarson et al. (1984) found that some varieties of soybeans do not respond well to high nitrogen fertilizer but variety Dunadjia (one of soybeans) had increased yield during the second year of a high nitrogen treatment. Mineral N may also inhibit nitrogenase activity and nodulation (Sprent et al., 1988).

Phosphorus is required for various molecular and biochemical plant processes (Epstein & Bloom, 2005), and it is critical for both N-fixers and the other plants as a major component of DNA and RNA, it also contributes to nodule development and seed production (Hellsten & Huss-Danell, 2000; Richmond & Davey, 2003; Vance et al., 2003). Nitrogen-fixing plants may have a higher requirement than other species for phosphorus and other nutrients (Sulieman et al., 2010). Because of the high requirement of ATP for nitrogen fixation by nitrogenase, phosphorus is particularly critical for nitrogen-fixing plants. Phosphorus also performs an important role in signal transduction, nodule

development and membrane biosynthesis that nitrogen fixers have an increased requirement for phosphorus than plants which receiving nitrogen fertilization directly from soil (Divito & Sadras, 2014; Graham & Vance, 2000). Also, the requirement of phosphorus supply may differ to different N-fixing plant species (Robson & Bottomley, 1991).

Apart from nutrient supply, soil pH also significantly influences nitrogen fixers. The growth of N-fixing plants may be reduced indirectly due to reduced nodulation in low pH soils. Some rhizobia tolerate lower soil pH but relatively few grow well below pH 4.5-5.0 (Mohammadi et al., 2012). The negative effects of soil acidity on plants and bacteria are due to a disruption of signal exchanges of symbionts and depression of nodulation genes (Mohammadi et al., 2012). Reflecting this, in one study Basu et al. (2008) reported that the leaf area index of peanut and dry matter production were significantly higher in plots with 2t ha^{-1} lime treatments than with no lime application.

Chapter 3

Isolation and identification of N-fixing and related bacteria from three exotic legumes (*Securigera varia*, *Astragalus cice*r and *Cytisus proliferus*)

3.1 Introduction

Biological nitrogen fixation is the process by which some bacteria fix atmospheric nitrogen and make it available to the plant. Leguminous and actinorhizal root systems are able to form nodules with their symbionts (rhizobia or Frankia) and fix nitrogen, in turn providing inputs of nitrogen to the ecosystem (Augusto et al., 2005; Franche et al., 2009). This symbiotic relationship has been widely exploited in agriculture. Leguminous crops such as soybean and pea have been extensively studied in many countries, including New Zealand (Feng et al., 1996; Peoples et al., 1995; Zahran, 1999). In this country Weir (2006) studied the ecology of rhizobia associated with native (*Carmichaelia*, *Clianthus* and *Sophora*) and exotic (*Acacia*, *Cytisus* and *Ulex*) species, and Tan (Tan, 2014) and Liu (Liu, 2014) characterised of the taxonomy of rhizobia associated with native species and common weed legumes. The range of species previously studied in New Zealand include the native genera of *Sophora*, *Carmichaelia*, *Montigena* and *Clianthus*, and exotic legume genera of *Robinia*, *Psoralea*, *Galega*, *Vicia*, *Medicago*, *Melilotus*, *Acacia*, *Lotus*, *Chamaecytisus*, *Lupinus*, *Ulex* and *Cytisus.* Directly relevant to the work in the present chapter, a small amount of research has focussed on the rhizobia colonizing *Cytisus proliferus* (Liu, 2014), but there is a paucity of information on the N-fixing bacteria colonizing *Securigera varia* and *Astragalus cice*r in New Zealand. These three N-fixing species have been used for soil rehabilitation, soil stabilization and forage in different countries (Gustine & Moyer, 1990; Heuzé et al., 2016; Holecheck et al., 1982).

The 16S ribosomal RNA (16S rRNA) is the most common gene used for construction of bacterial phylogenies and taxonomy studies due to the slow evolution rates of this gene (Clarridge, 2004). This gene encodes a small subunit of prokaryotic ribosomes (Schluenzen et al., 2000). Additionally, some conserved housekeeping genes such as ATP synthase beta-subunit (*atpD*) and recombinase A (*recA*) have been used for phylogeny studies of rhizobia (Gaunt et al., 2001). Also, genes involved in nitrogen fixation (*nif* and *fix*) and nodulation (*nod, nol* and *noe*), which are essential for establishing an effective symbiosis, have been used to identify and classify rhizobia (Fischer, 1994; Laranjo et al., 2014).

The work presented in this chapter focussed on analytical techniques and protocols. The selected species were those considered to potentially have a role in the early stages of ecological restoration of vegetation. The aim of the work presented in this chapter was to isolate the nitrogen-fixing related bacteria from the three exotic legumes *Securigera varia*, *Astragalus cice*r and *Cytisus proliferus*. Once recovered into culture, bacteria were identified using the sequence of the 16S rRNA gene and the *recA* or *nifH* gene.

3.2 Methods

3.2.1 Nodule collection and strain isolation

Securigera varia plants (approximate one-year old) were randomly collected in a field planted with this species at Woodend, Canterbury (-43.342, 172.669) in 2014. Ten nodules collected from 5 of plants (*Securigera varia*) were used for isolation. Seeds of *Securigera varia* and *Astragalus cice*r were collected (by Richard Lucas) from multiple and mature plants in 2012 and 2014 respectively. Seeds of *Cytisus proliferus* were collected from multiple and mature plants (by Richard Lucas in 2013) at the hedge on the south side of Iversen Field on the Lincoln University campus (-43.648, 172.466). Thirty of the seeds from *Astragalus cice*r and *Cytisus proliferus* were scarified and soaked in water for overnight then sown into two different pots by species with natural soil collected from a dairy farm (-43.648, 172.464) about 0 to 15 cm depth. Twelve nodules were then collected from 6 plants for each species (*Astragalus cice*r and *Cytisus proliferus*), excavated from the pots, to be used for bacterial isolation.

The roots were washed under tap water and the nodules were excised from roots using sterilized scissors. Each nodule was immersed in 96% ethanol for 5 -10 seconds in a laminar flow cabinet. After that, the nodules were transferred into 1% - 2% sodium hypochlorite solution for 3 - 5 minutes for surface sterilization followed by a sterilized water rinse several times. The nodules were then dissected by scalpel and the isolates from the nodules were streaked onto Petri plates containing yeast mannitol agar (YMA) media (Appendix **A**) (Vincent, 1970) using inoculating loops. The Petri dishes were incubated at 25°C for 3 to 5 days in dark conditions.

3.2.2 Purification and DNA extraction

Single colonies (circular, convex, raised and mucilaginous) were selected from the original Petri dishes then sub-cultured onto new YMA plates, this step was repeated 2-3 times to obtain a pure culture. Pure cultures were stored in YMB fluid medium consisting 20% glycerol at -80°C for long-

term storage. Pure strains from single colonies were inoculated separately in 1000 ul centrifugation tubes with 700 ul of YMB media (Appendix A) for DNA extraction.

DNA was extracted from bacterial cultures using the PureGene DN extraction kit, QIAGEN according to the manufacturer's instructions. The quality and quantity of DNA in each extraction was measured using a spectrophotometer (NanoDrop™) at 260 and 280 nm. DNA samples were diluted to 50 ng uL^{-1} for PCR.

3.2.3 PCR amplification

The technique of polymerase chain reaction (PCR) was used to amplify the 16S rRNA, *recA* and *nifH* genes. The primers used to amplify 16S rRNA were F27 (5'- AGA GTT TGA TCM TGG CTCAG-3') (Lagacé et al., 2004) and R1494 (5'- CTA CGG YTA CCT TGT TAC GAC-3') (Gomes et al., 2001)), *recA* were 41F (5'- TTC GGC AAG GGM TCG RTS ATG-3'), 640R (5'- ACA TSA CRC CGA TCT TCA TGC-3') (Vinuesa et al., 2005)) and *nifH* were Pol F (5'TGCGAYCCSAARGCBGACTC-3') and Pol R (5'-ATSGCCATCATYTCRCCGGA-3') (Poly et al., 2001)). Each PCR contained 2.5 μl 10× PCR Buffer, 2.0 μl of 2.5 mM dNTPs, 1μl of 10 μM of each forward and reverse primer, 0.25 μl of 5U ul^{-1} FastStart Taq Polymerase (Roche), 50 ng genomic DNA and 17.25 μl distilled water. PCR conditions were 95˚C for 3 minutes then 35 cycles of 94˚C for 30 seconds, 55 -65˚C for 30 -40 seconds, 72˚C for 1 minute, 72˚C for 7 minutes then 4˚C for storage.

3.2.4 DNA analysis

PCR products were separated by 1% agarose gel electrophoresis at 100 -120V for 30-40 min and visualized under UV light after staining with 0.5 μg mL^{-1}ethidium bromide for 10 minutes. PCR products were sequenced directly at the Bio-Protection Research Centre, Lincoln University after confirmation by gel electrophoresis. Sequencing results were analyzed by biology software (Sequence Scanner; DNAMAN, Lynnon Biosoft). Phylogenetic trees were analyzed by MEGA6 software (MEGA6 SERVICES LTD, version 6.06). Additional DNA sequences presented in phylogenetic tree were obtained from GenBank (http://blast.ncbi.nlm.nih.gov/).

3.2.5 Inoculation test

Thirty seeds from each species of *Securigera varia*, *Astragalus cice*r and *Cytisus proliferus* were scarified using a file and surface sterilized in 70% ethanol for two minutes then rinsed several times with sterile water. After that, seeds were soaked in warm sterile water at room temperature for 3-5 days for germination. Three replicates of the germinated seedlings from each species were grown in

treated containers (50ml PET jar, UV for 1 hour) with 35ml autoclaved fine grade vermiculite. Each container contained one of the seedlings. All the seedlings grown in a controlled-environment cabinet with 12 hours light at 25˚C for 10 weeks. Complete nutrient solution (Liu, 2014) containing NH_4NO_3 (0.1 mM), $CaCl_2$ (1.0 mM), KCl (1.0 mM), $MgSO_4.7H_2O$ (1.0 mM), NaH_2PO_4 (1.0 mM), Na_2HPO4 (0.1 mM), $FeCl_2.4H_20$ (5.0 µM), H_3BO_3 (5.0 µM), $MnCl_2.2H_2O$ (1.0 µM), $Na_2MoO_4.2H_20$ (0.5 µM), $CuSO_4.5H_2O$ (0.1 µM), $ZnSO_4.7H_2O$ (0.1 µM) and $CoCl_2.6H_2O$ (0.02 µM) was prepared to maintain the growth of seedlings. Thirty mL of the nutrient solution was added into each container containing vermiculite and a germinated seed. The solution (30ml) was reapplied every 2 weeks. Three of seedlings from each species were set up as control group.

For inoculation of plants, a total 13 isolates were grown in 50 ml of YMB in a shaking incubator at 100 rpm at 25°C for 3 to 5 days (approximately $4*10^8$ cfu ml^{-1}). Ten mL of each culture was inoculated to each of the seedling 2 weeks after the seeds were sown in the container. To the control group (3 replicates of each species) 10 mL of YMB media without bacteria was added.

3.3 Results

3.3.1 Phylogenetic analyses of the 16S rRNA and *nifH* genes of the isolates from *Securigera varia*.

Four isolates were identified as *Mesorhizobium spp*. by 16s rRNA sequences (1328- 1354 bp) (Figure 3.1). The four isolates (C1, C2, C3, C4) were in the same group and 99% similar to each other. The most similar named species was *Mesorhizobium caraganae* CCBAU 11299 (1365 bp, 97% - 98.61% similarity). The isolated strains were more similar to strains isolated from New Zealand native plants than to those isolated from exotic plants. In addition, the nearest cluster contained *Mesorhizobium* spp. that are associated with the native species *Clianthus puniceus*, *Carmichaelia nana* and *Carmichaelia odorata* were close to the *Mesorhizobium huakuii* type strain. *Rhizobium pisi* DSM 30132 type strain was used as an outgroup.

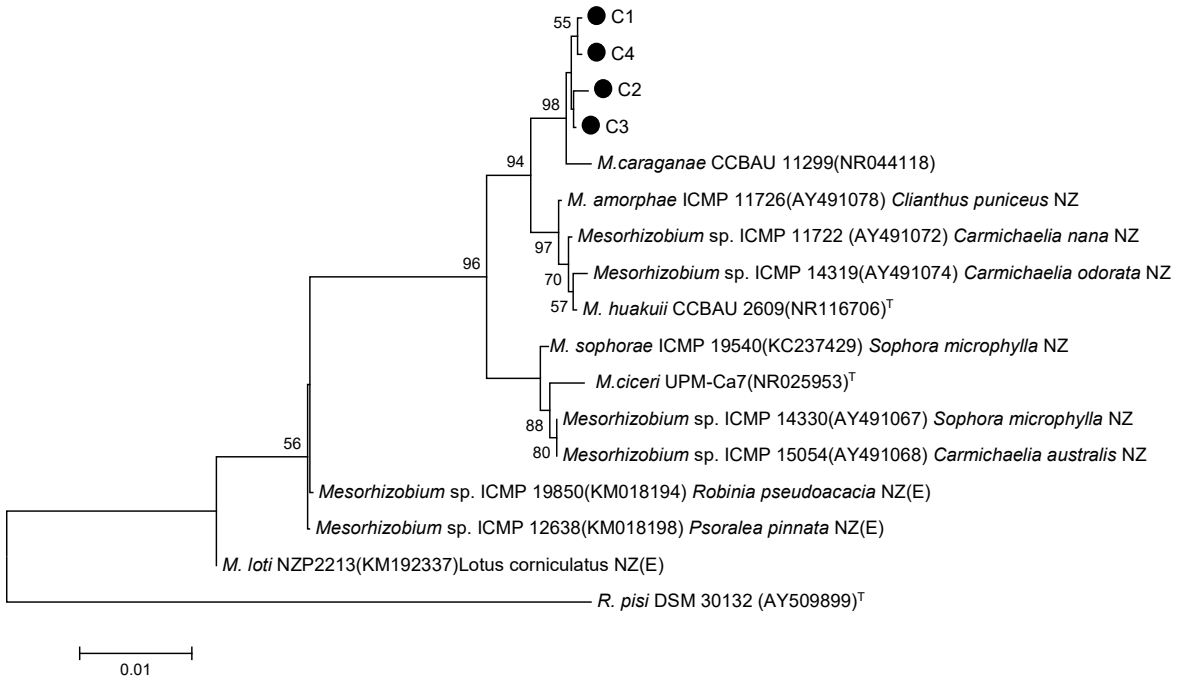

Figure 3.1 Phylogenetic tree of 16S rRNA gene sequences of four bacterial isolates from *Coronilla varia* (syn. *Securigera varia*) sampled in New Zealand (●), selected *Mesorhizobium* and *Rhizobium* spp. type strains and *Mesorhizobium* spp. associated with New Zealand native (NZ) and exotic (NZ(E)) legumes. 'T' indicates type strain. M. = *Mesorhizobium*, R. = *Rhizobium*, B. = *Bradyrhizobium*. *Rhizobium pisi* DSM 30132T was used as outgroup. This tree was constructed using the MEGA6 software with the Neighbor-joining Tamura 3-parameter method. GenBank accession numbers are in parentheses. Numbers on branches are bootstrap % from 1000 replicates (shown only when ≥ 50%). Scale bar represents 0.01 amino acid substitutions per site (1 substitution per 100 nucleotides).

Alignment of the *nifH* gene sequences (306-315bp) showed the isolated strains (C1, C2, C3, C4) from *Coronilla varia (syn. Securigera varia)* were 95.45% similar to each other and formed a phylogenetic group with *Mesorhizobium sp.* CCNWSX661 (Figure 3.2) which was isolated from *Securigera varia* in China. The four isolated strains were also similar to *Mesorhizobium* sp. WSM2075 type strain and *M. cicer* type strain.

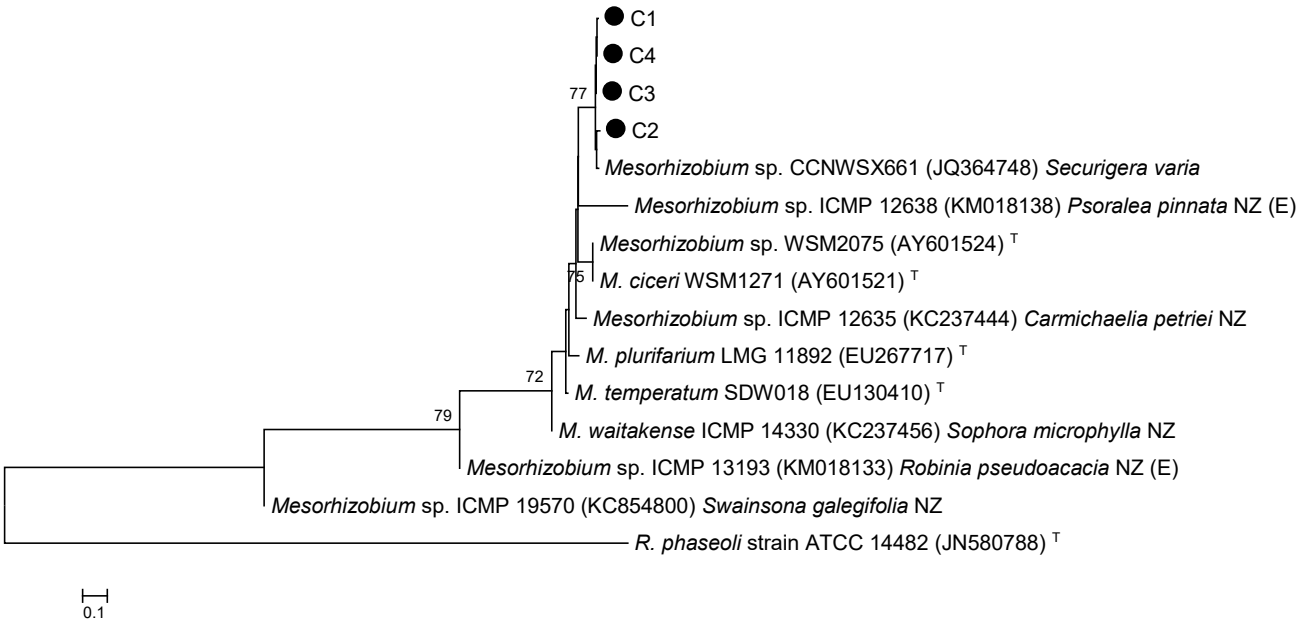

Figure 3.2 Phylogenetic tree of *nifH* gene sequences of four bacterial isolates from *Coronilla varia (Securigera varia)* sampled in New Zealand (●), selected *Mesorhizobium* and *Rhizobium* spp. type strains and *Mesorhizobium* spp. associated with New Zealand native (NZ) and exotic (NZ(E)) legumes. 'T' indicates type strain. M. = *Mesorhizobium*, R. = *Rhizobium*. *Rhizobium phaseoli* strain ATCC 14482T was used as outgroup. This tree was constructed using the MEGA6 software with the Neighbor-joining Tamura 3-parameter method. GenBank accession numbers are in parentheses. Numbers on branches are bootstrap % from 1000 replicates (shown only when ≥ 50%). Scale bar represents 0.1 amino acid substitutions per site (1 substitution per 10 nucleotides).

3.3.2 Phylogenetic analyses of 16S rRNA, *recA* and *nifH* genes of the isolates recovered from *Astragalus cice*r.

The phylogenetic analysis of 16S rRNA showed that the four bacterial strains obtained from *Astragalus cice*r formed two groups (Figure 3.3). Strains A1 and A3 (669 bp, 669 bp; 96.77% similarity) were *Mesorhizobium* spp. and strains A2 and A5 (711 bp, 703 bp; 97.61% similarity) were *Ochrobactrum* spp.. Isolates A1 and A3 were most similar to the *Mesorhizobium robiniae* type strain based on 16S rRNA sequences. A1 and A3 were also similar to *Mesorhizobium tianshanense* W59 and *Mesorhizobium sp.* which were isolated from *Astragalus scaberrimus* and *Glycyrrhiza uralensis,* in China and Finland respectively. A2 was most similar to *Ochrobactrum* sp. 112 isolated from soil in Turkey (710 bp, 98.87% similarity). A5 was similar to *Ochrobactrum rhizosphaerae* W46 isolated

from Liangshui river in China (706 bp, 98.59% similarity).

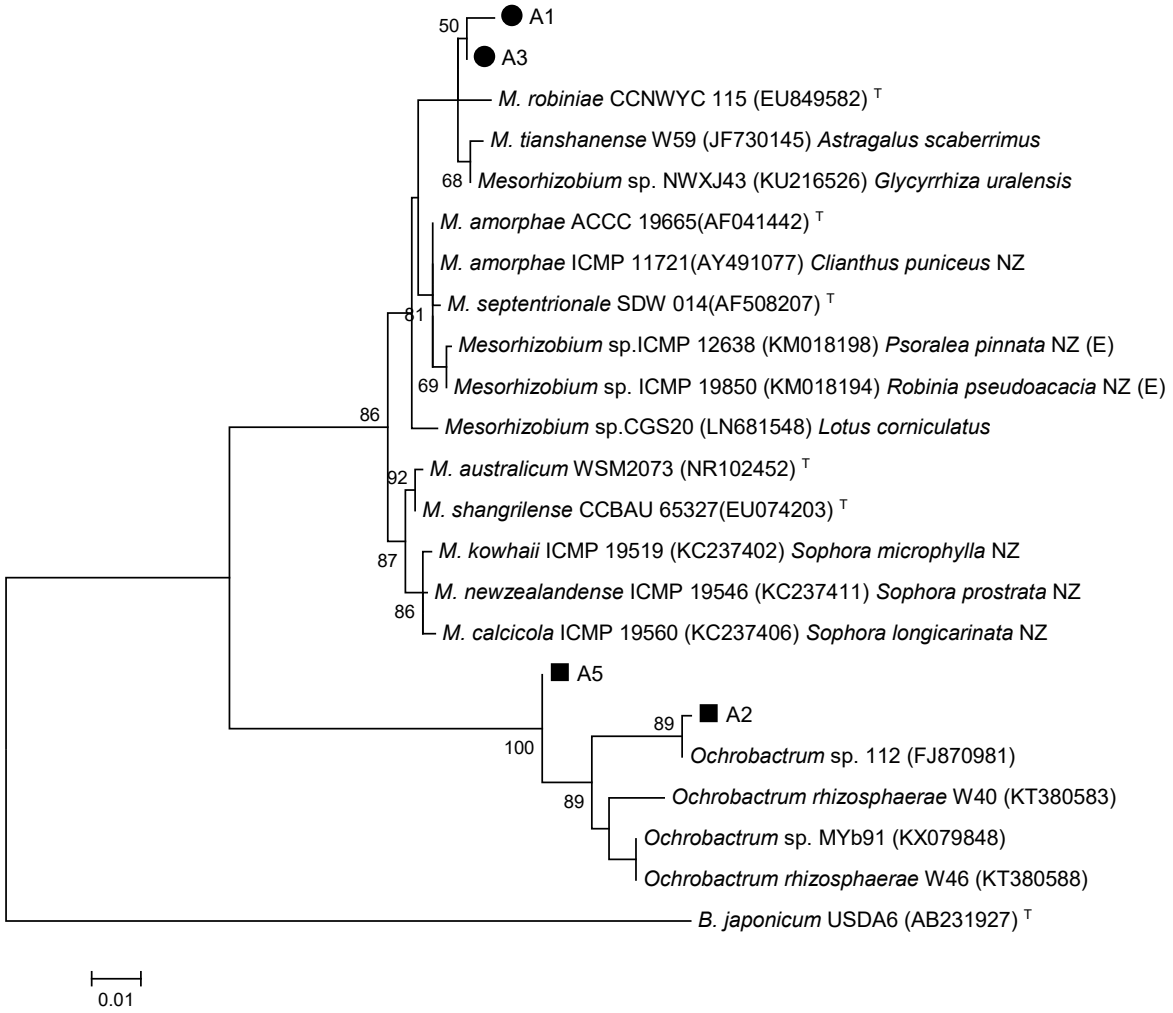

Figure 3.3 Phylogenetic tree of 16S rRNA gene sequences of four bacterial isolates from *Astragalus cicer* root nodules sampled in New Zealand (● /▪), selected *Mesorhizobium* type strains, *Mesorhizobium* spp. associated with New Zealand native (NZ) and exotic (NZ(E)) legumes and *Ochrobactrum* spp.. 'T' indicates type strain. M. = *Mesorhizobium*, B. = *Bradyrhizobium*. *Bradyrhizobium japonicum* USDA6T was used as outgroup. This tree was constructed using the MEGA6 software with the Maximum likelihood Tamura 3-parameter Gamma distributed with Invariant sites (G+I) method. GenBank accession numbers are in parentheses. Numbers on branches are bootstrap % from 1000 replicates (shown only when ≥ 50%). Scale bar represents 0.01 amino acid substitutions per site (1 substitution per 100 nucleotides).

Phylogenetic analysis of *recA* gene sequences also showed the four isolates isolated from *Astragalus cice*r divided into two groups. Isolates A1 and A3 (279, 270 bp; 96.06% similarity) were placed in the *Mesorhizobium* group. A2 and A5 (483, 438 bp; 90.68% similarity) were placed within the *Ochrobactrum* spp.. The phylogenetic tree (Figure 3.4) showed A1 and A3 were most similar to

Mesorhizobium metallidurans STM3973 that was isolated from *Anthyllis vulneraria* in Europe. A2 and A5 were most similar to the *Ochrobactrum anthropi* CCUG 50899 type strain (440 bp, 99% similarity).

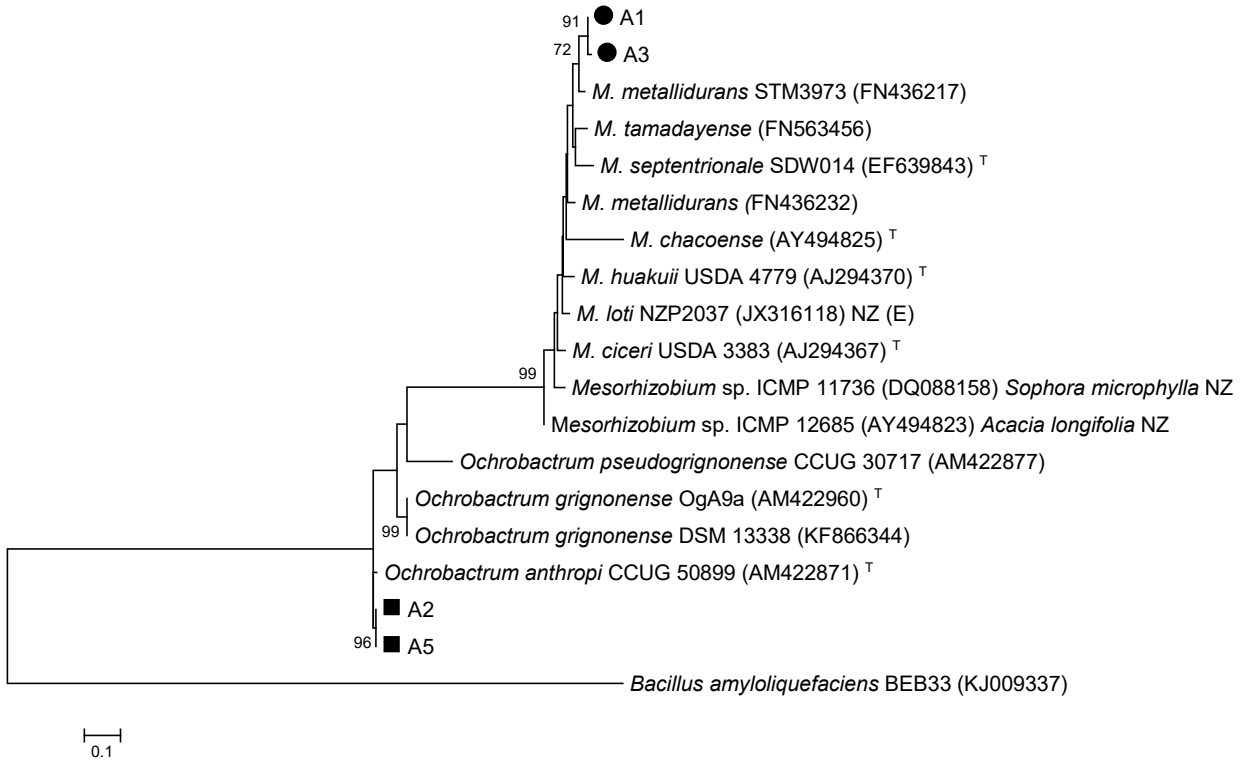

Figure 3.4 Phylogenetic tree of *recA* gene sequences of four bacterial isolates from *Astragalus cicer* root nodules sampled in New Zealand (● /▪), selected *Mesorhizobium* type strains, *Mesorhizobium* spp. associated with New Zealand native (NZ) and exotic (NZ(E)) legumes and *Ochrobactrum* spp.. 'T' indicates type strain. M. = Mesorhizobium. *Bacillus amyloliquefaciens* BEB33 was used as outgroup. This tree was constructed using the MEGA6 software with the Maximum likelihood Tamura 3-parameter Gamma distributed with Invariant sites (G+I) method. GenBank accession numbers are shown in parentheses. Numbers on branches are bootstrap % from 1000 replicates (shown only when ≥ 50%). Scale bar represents 0.1 amino acid substitutions per site (1 substitution per 10 nucleotides).

In contrast, the *nifH* gene sequences analyses placed all four isolates (A1, A2, A3, A5) in the same cluster with *Mesorhizobium* amorphae CCNWX667 and *Mesorhizobium* sp. CCNWX661 isolated from *Securigera varia* in China (Figure 3.5). The four strains showed 98.05% similarity to each other and the length of the *nifH* sequences was from 286 to 291bp. Figure 3.5 showed the *nifH* genes from the four isolates also close to *Mesorhizobium* sp. ACMP18 which were obtained from *Astragalus cicer* in Poland. The two selected *nifH* gene sequences from *Ochrobactrum* spp. were distributed within the groups of *Mesorhizobium*.

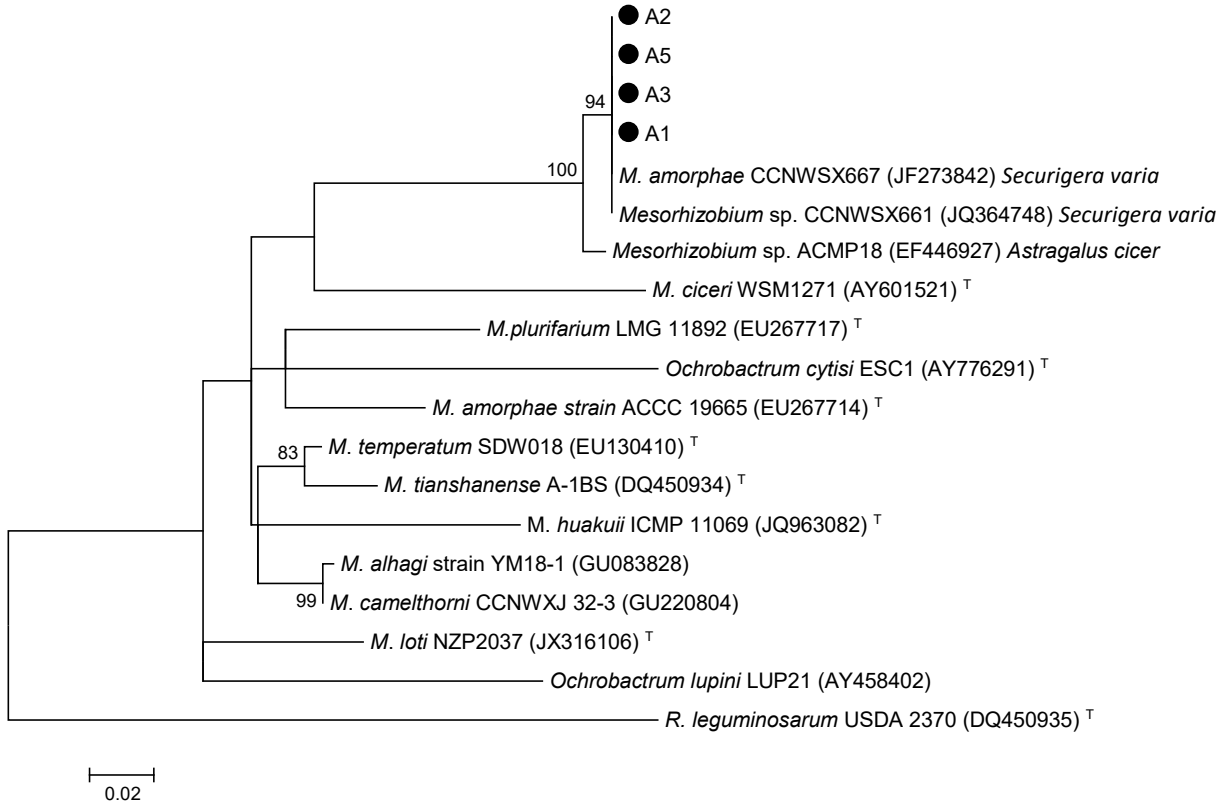

Figure 3.5 Phylogenetic tree of *nifH* gene sequences of the four bacterial isolates from *Astragalus cicer* root nodules sampled in New Zealand (●), selected *Mesorhizobium* and *Rhizobium* spp. type strains, other *Mesorhizobium* spp. and *Ochrobactrum* spp.. 'T' indicates type strain. M. = *Mesorhizobium*, R. = *Rhizobium*. *Rhizobium Leguminosarum* USDA 2370T was used as outgroup. This tree was constructed using the MEGA6 software with the Maximum likelihood Tamura 3-parameter Gamma distributed with Invariant sites (G+I) method. GenBank accession numbers are in parentheses. Numbers on branches are bootstrap % from 1000 replicates (shown only when ≥ 50%). Scale bar represents 0.02 amino acid substitutions per site (2 substitution per 100 nucleotides).

3.3.3 Phylogenetic analyses of 16S rRNA, *recA* and *nifH* genes of the isolates from *Cytisus proliferus*.

Five isolates from *Cytisus proliferus* were placed into two groups by their 16S rRNA sequences (Figure 3.6). Three isolates (T8, T9, T10) were placed in the same group (1220-1230 bp, 98.41% similarity). They were most similar to *Bradyrhizobium cytisus* ICMP 19829 strain (1314 bp, 91.82% similarity) previously isolated from *Cytisus scoparius* present in New Zealand. The three isolates were also similar to some *Bradyrhizobium* type strains including *B. japonicum* USDA6, *B. canariense* BTA-1 and *B. daqingense* CCBAU 15774. Two of the isolates (T7, T12) were *Ochrobactrum* spp. which

were similar to *Ochrobactrum pituitosum* SPT1-119a strain isolated from *Ammopiptanthus* root nodule in China (Figure 3.6).

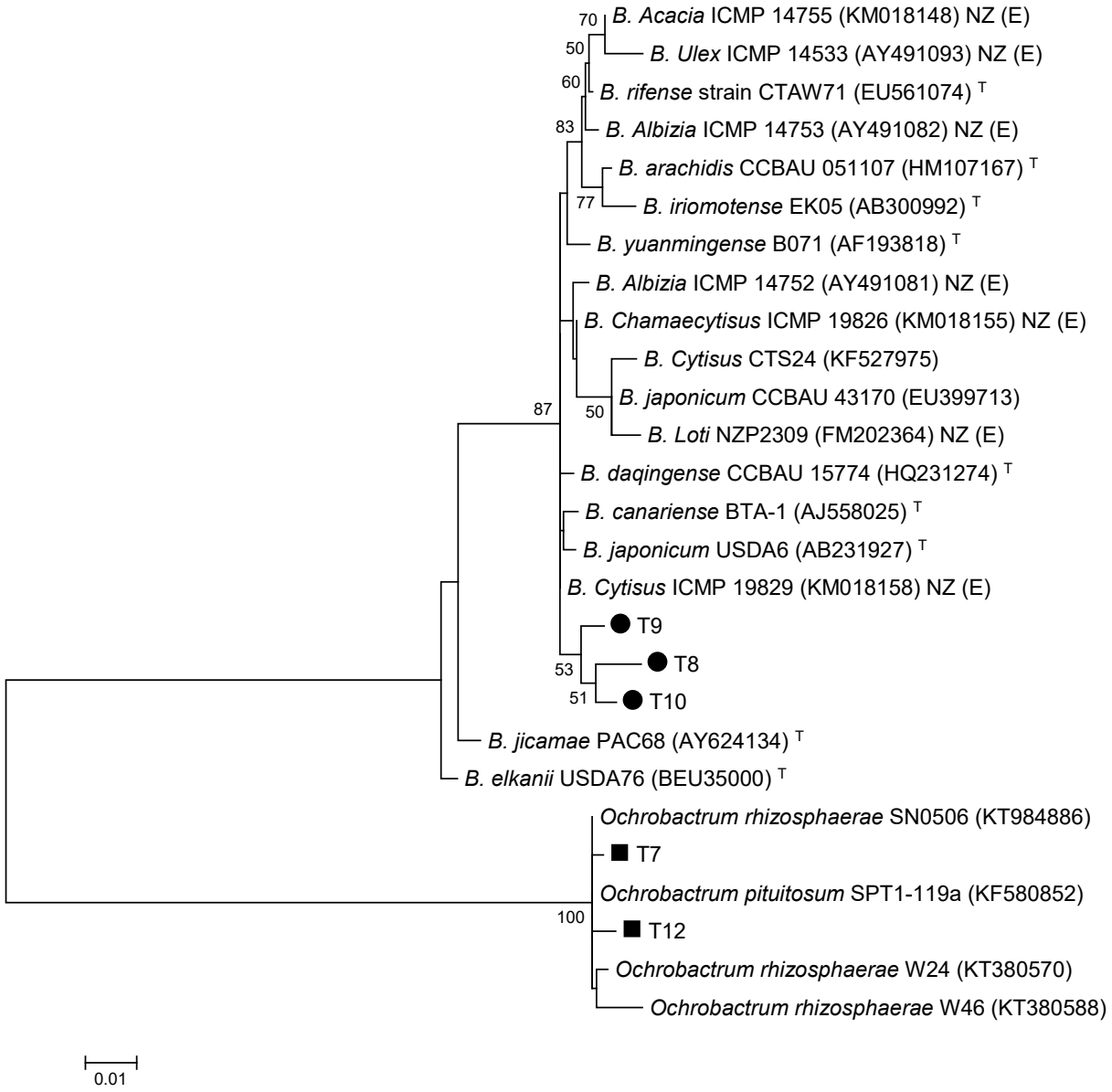

Figure 3.6 Phylogenetic tree of 16S rRNA gene sequences of five bacterial isolates from *Cytisus proliferus* root nodules sampled in New Zealand (● /▪), selected *Bradyrhizobium* type strains, *Bradyrhizobium* spp. associated with New Zealand exotic (NZ(E)) legumes and other *Bradyrhizobium* and *Ochrobactrum* spp.. 'T' indicates type strain. B. = *Bradyrhizobium*. This tree was constructed using the MEGA6 software with the Maximum likelihood Tamura 3-parameter Gamma distributed with Invariant sites (G+I) method. GenBank accession numbers are in parentheses. Numbers on branches are bootstrap % from 1000 replicates (shown only when ≥ 50%). Scale bar represents 0.01 amino acid substitutions per site (1 substitution per 100 nucleotides).

In terms of *recA* gene sequences analyses, the five isolates from *Cytisus proliferus* were also placed into the same two groups. Isolates T8, T9 and T10 (322, 323, 323 bp, 99.82% similarity) were grouped together in *Bradyrhizobium* cluster (Figure 3.7). Isolates T9 and T10 were most similar to *Bradyrhizobium* sp. ICMP 19831 and 14291 associated with *Cytisus scoparius* presented in New Zealand. Isolate T8 was most similar to *Bradyrhizobium* sp. muu.1a, isolated from *Mucuna urens*. Isolates T7 (299 bp) and T12 (452 bp) were most similar to the *Ochrobactrum anthropic* CCUG 50899 type strain.

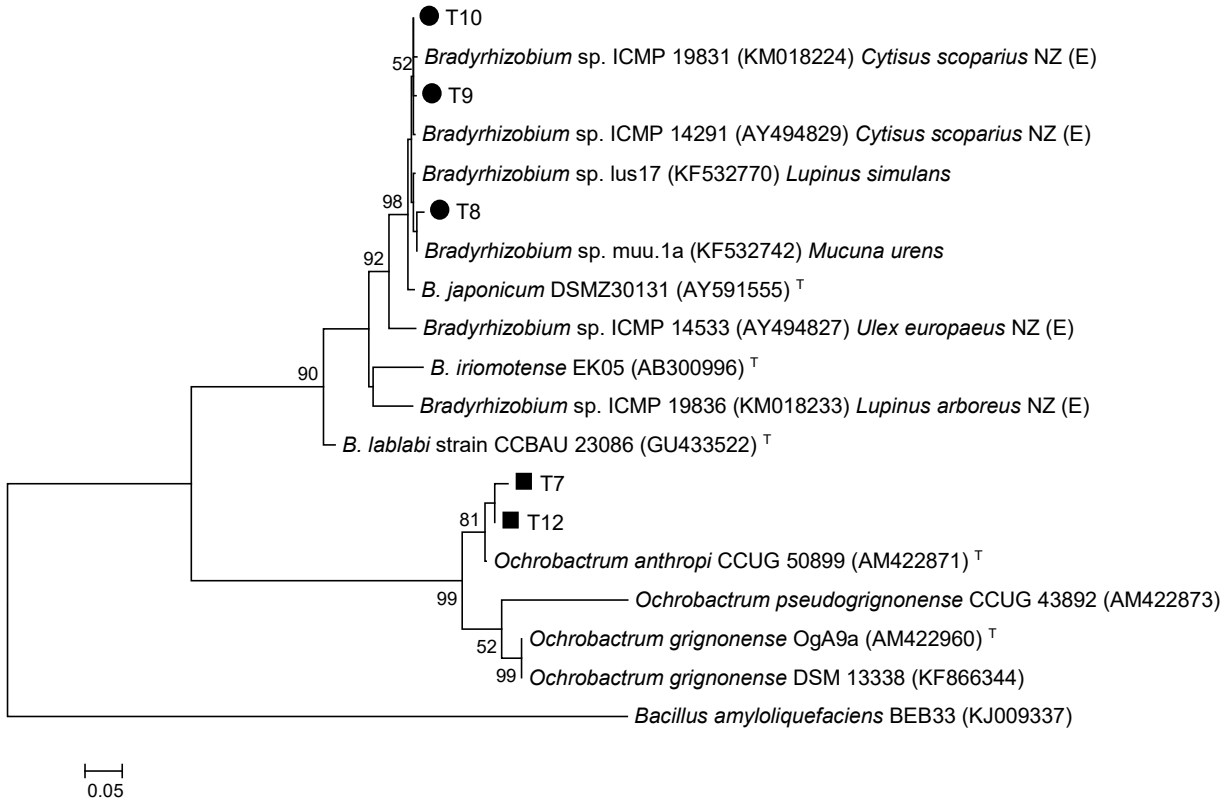

Figure 3.7 Phylogenetic tree of *recA* gene sequences of five bacterial isolates from *Cytisus proliferus* root nodules sampled in New Zealand (● /▪), selected *Bradyrhizobium* type strains, *Bradyrhizobium* spp. associated with New Zealand exotic (NZ(E)) legumes and other *Bradyrhizobium* and *Ochrobactrum* spp.. 'T' indicates type strain. B. = Bradyrhizobium. *Bacillus amyloliquefaciens* BEB33 was used as outgroup. This tree was constructed using the MEGA6 software with the Maximum likelihood Tamura 3-parameter Gamma distributed with Invariant sites (G+I) method. GenBank accession numbers are in parentheses. Numbers on branches are bootstrap % from 1000 replicates (shown only when ≥ 50%). Scale bar represents 0.05 amino acid substitutions per site (5 substitution per 100 nucleotides).

Unlike the 16S rRNA and *recA* gene sequences, the *nifH* gene sequences of the five isolates were similar (255-187bp, 93.66% similarity) and placed them in the same group with *Bradyrhizobium* spp. (Figure 3.8). Isolates of T9 and T10 were most simialr to *Bradyrhizobium* sp. ICMP 191831 which was isolated from exotic species *Cytisus scoparius* in New Zealand. Isolates T8 and T12 were most similar to *Bradyrhizobium* sp. ZAR2 which associated with *Sarothamnus scoparius*, and isolate T7 was most similar to the *Bradyrhizobium cytisi* CTAW11 type strain. Selected *Ochrobactrum* spp. from GenBank were placed in a group with the *Rhizobium leguminosarum* USDA 2370 type strain.

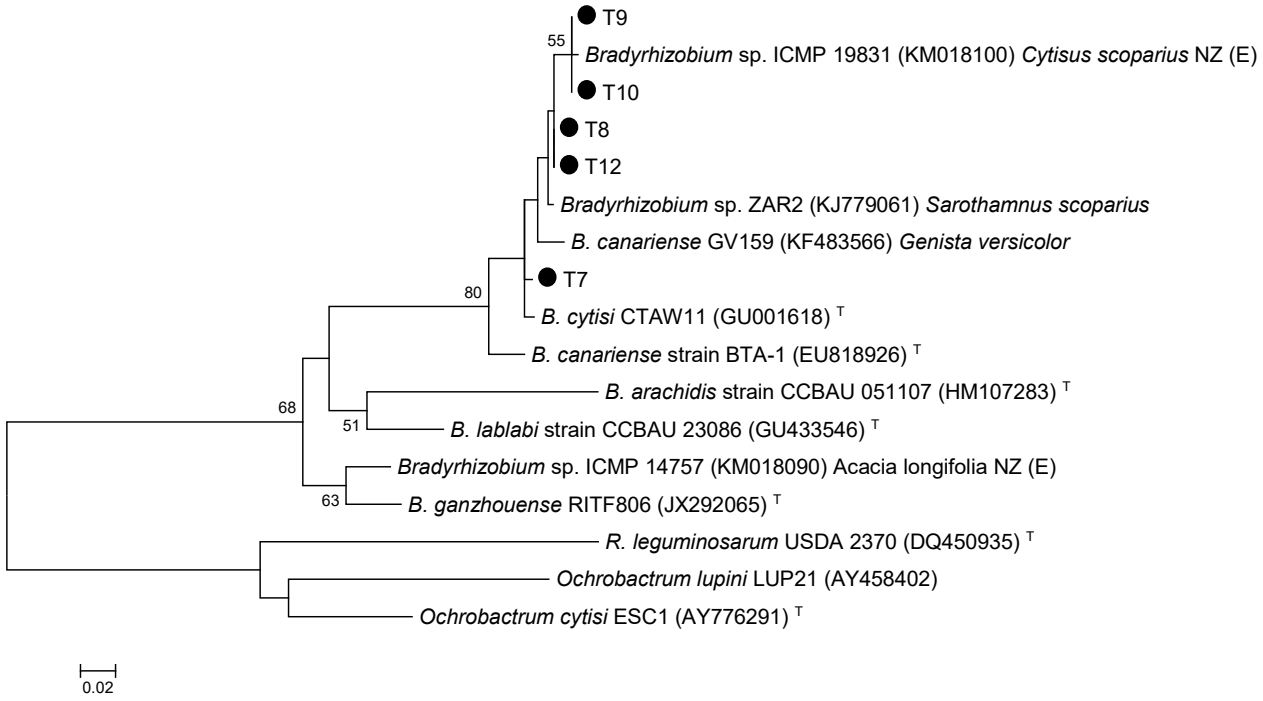

Figure 3.8 Phylogenetic tree of *nifH* gene sequences of five bacterial isolates from *Cytisus proliferus* root nodules sampled in New Zealand (●), selected *Bradyrhizobium* and *Ochrobactrum* type strains, *Bradyrhizobium* spp. associated with New Zealand exotic (NZ(E)) legumes and other *Bradyrhizobium* and *Ochrobactrum* spp.. 'T' indicates type strain. B. = Bradyrhizobium, R. = Rhizobium. *Rhizobium leguminosarum* USDA 2370T was used as outgroup. This tree was constructed using the MEGA6 software with the Maximum likelihood Tamura 3-parameter Gamma distributed with Invariant sites (G+I) method. GenBank accession numbers are in parentheses. Numbers on branches are bootstrap % from 1000 replicates (shown only when ≥ 50%). Scale bar represents 0.02 amino acid substitutions per site (2 substitution per 100 nucleotides).

3.3.4 Inoculation test

The isolates of C1, C2, C3 and C4 which isolated from *Securigera varia* root nodules and identified as *Mesorhizobium spp*. were inoculated for *Securigera varia* seedlings. Isolates A1, A3 (*Mesorhizobium spp*.) and A2, A5 (*Ochrobactrum* spp.) isolated from *Astragalus cicer* were inoculated for *Astragalus cicer* seedlings. Isolates T8, T9, T10 (*Mesorhizobium spp*.) and T7, T12 (*Ochrobactrum* spp.) isolated from *Cytisus proliferus* root nodules were inoculated for *Cytisus proliferus* seedlings. Inoculation results showed all the isolates could form nodules with their host plant species (Figure 3.9) whereas control (C) plants were not nodulated.

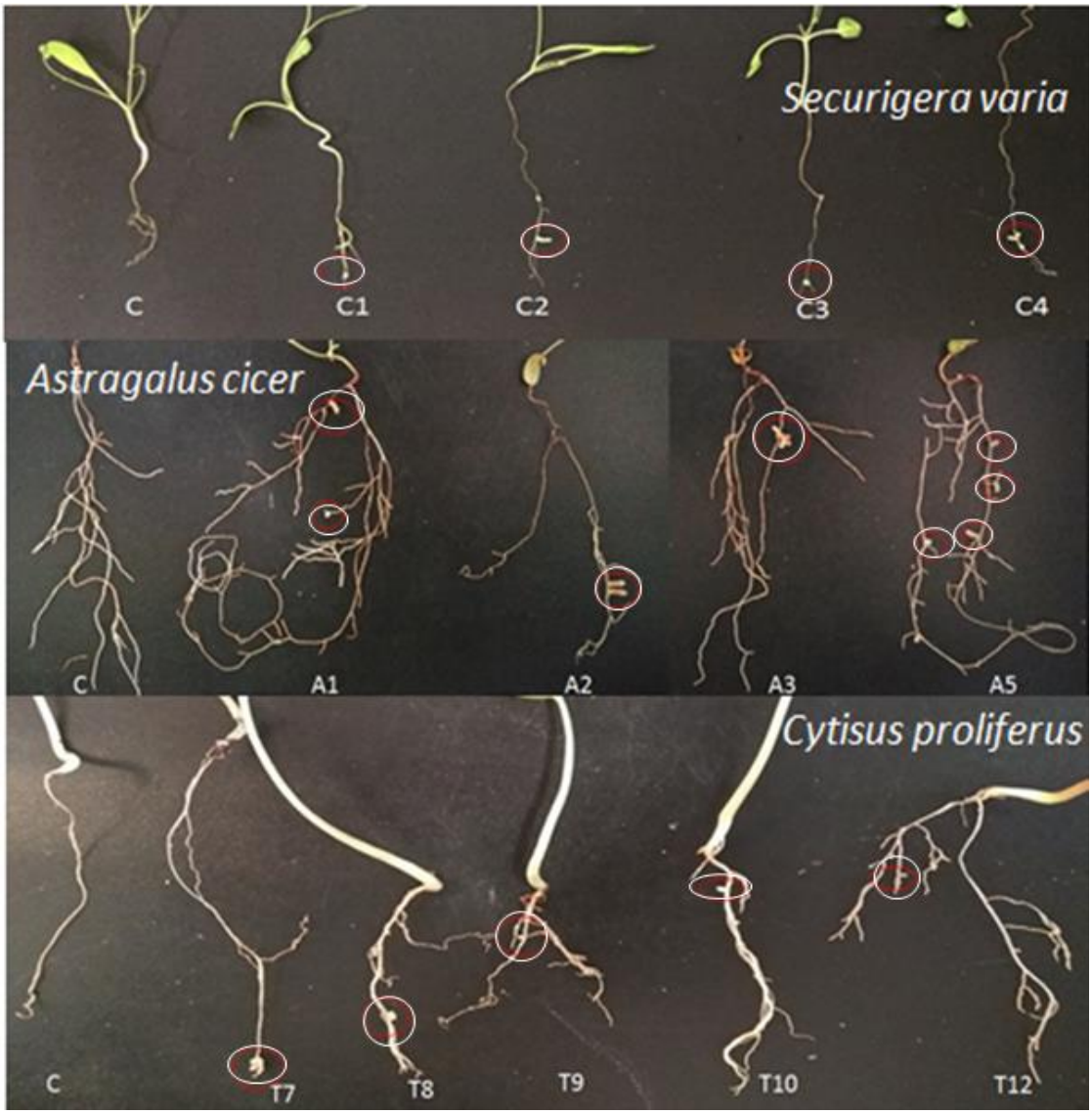

Figure 3.9 Inoculation test of the three exotic nitrogen-fixing species. 'C' indicates control plants which were not inoculated by any bacteria. Plant C1 to C4, A1-A5 and T7-T12 were inoculated by their corresponding isolates. Root nodules shown in white circles.

3.4 Discussion

In total, thirteen isolates were recovered from the three legumes species present in New Zealand as exotic species; four isolates from *Securigera varia*, four from *Astragalus cicer* and five from *Cytisus proliferus*. Four of the isolates from *Securigera varia* were identified as *Mesorhizobium* spp. in terms of 16S rRNA gene sequences analyses. Two of the four isolates which isolated from *Astragalus cicer* were identified as *Mesorhizobium* spp. and the other two were *Ochrobactrum* spp.. Three of the five isolates which isolated from *Cytisus proliferus* were identified as *Bradyrhizobium* spp. and the other two were *Ochrobactrum spp.*.

Rhizobium, *Ensifer*, and *Mesorhizobium* are classified as fast-growing rhizobia (Boukhatem et al., 2012). Rhizobia can be also classified according to the host legumes which they can nodulate (cross-inoculation groups) (Greenwood & Pankhurst, 1977). For example, Greenwood and Pankhurst (1977) reported sainfoin, crown and vetch can be classified into a sainfoin "cross-inoculation groups" associated with fast-growing rhizobia. *Mesorhizobium* spp. (fast-growing rhizobia) were isolated from *Securigera varia* (crown vetch) and *Astragalus cicer* (cicer milkvetch) in New Zealand, in the present study, indicating these two species may be classified into Sainfoin "cross-inoculation groups" which are associated with fast-growing rhizobia.

There was no prior report of gene phylogenies of rhizobia isolated from *Securigera varia* present in New Zealand. Wenquan et al. (2013) investigated the genetic diversity of rhizobia isolated from *Securigera varia* in Shanxi province, China. In total, they recovered 90 isolates that belong to six different genotypes (Figure 3.10). They were distributed into three genera (*Mesorhizobium*, *Rhizobium* and *Agrobacterium*) according to the 16S rRNA analysis, and 86.7% of all the isolates were characterized as *Mesorhizobium* spp. The isolates which were obtained from *Securigera varia* in New Zealand all belonged to *Mesorhizobium* spp. in terms of their 16S rRNA gene sequences. This indicates that *Mesorhizobium* is the most dominant species which symbiotic with *Securigera varia*. There was, however, some differences between the isolates from New Zealand *Securigera varia* and China. The 16S rRNA analysis from Wenquan et al. (2013) showed 66.7% of isolates were from two genotypes (CCNWSX662, CCNWSX672; Figure 3.10) closely related to *M. alhagi* whereas, the isolates from Canterbury, New Zealand were more similar to *M. caraganae*. *Securigera varia* with beautiful pink flowers is native to Europe and Asia, but very rare to present in New Zealand. The result from this current study evidence *Securigera varia* is able to associate with *Mesorhizobium spp.* to form nodules in New Zealand. It would be interesting to investigate if *Rhizobium* and *Agrobacterium* could be symbiotic with *Securigera varia* in New Zealand.

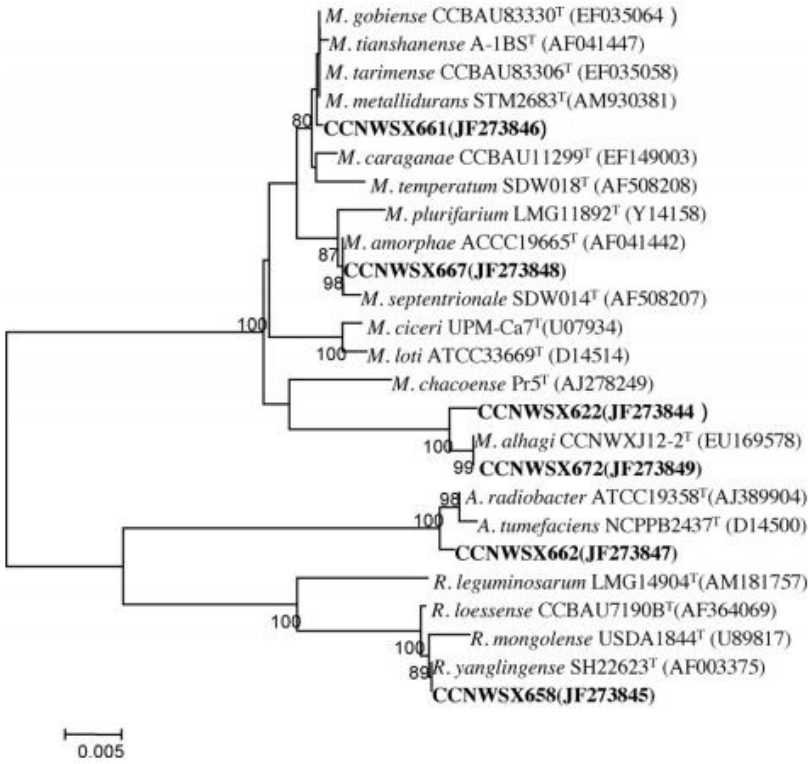

Figure 3.10 Phylogenetic tree based on 16S rRNA gene sequences using the neighbour-joining method. The figures on the branches indicate the reliabilities. GeneBank accession numbers are in parenthesis. The strains in boldface were used for 16S rRNA gene sequencing. The scale bar indicates the number of substitutions per site (Wenquan et al., 2013).

There was no previous report for rhizobia isolated from *Astragalus cicer* in New Zealand. Wdowiak and Małek (2000) studied 37 isolates isolated from *Astragalus cicer* in Canada, Ukraine and Poland and showed they were related to *Mesorhizobium* species and located in two major clusters. One was the *Mesorhizobium loti* branch containing the rhizobia from Poland and the other was similar to *M. tianshanense, M. mediterraneum, M. ciceri, and M. huakuii* and comprised the isolates from Canada, Ukraine and one isolate from Poland. Wdowiak-Wróbel and Małek (2005) investigated 36 *Astragalus cicer* nodule isolates and 9 reference mesorhizobia using the amplified fragment length polymorphism (AFLP) method and found a similar result to the early research (Wdowiak & Małek, 2000). Two of the isolates from *Astragalus cicer* grown in New Zealand soil were identified as *Mesorhizobium* spp. and they were most closely related to *M. tianshanense* which is same as some of the isolates obtained from *Astragalus cicer* in Canada and Europe.

Some studies reported that *Bradyrhizobium* spp. are able to nodulate *Cytisus proliferus* (Liu, 2014; Vinuesa et al., 1998). Five *Bradyrhizobium* species have been isolated from *Cytisus proliferus* in New Zealand in a previous work (Liu, 2014). In terms of 16S rRNA gene analysis, two of the five isolates

from Liu's study were similar to a *Bradyrhizobia* sp. isolated from *Cytisus scoparius* in New Zealand, a *Bradyrhizobia* sp. isolated from *Genista tinctoria* in the UK and the other three were similar to *Bradyrhizobium* spp. isolated from *Ulex europaeus* and *Lotus pedunculatus* in New Zealand. In the present study, the isolates from *Cytisus proliferus* were close to *Bradyrhizobium* sp. isolated from *Cytisus scoparius* in New Zealand in terms of phylogenetic analysis of 16S rRNA genes. From the results of previous work and this research, most of the *Bradyrhizobium* species isolated from *Cytisus proliferus* in New Zealand were similar to *Bradyrhizobium* spp. isolated from New Zealand exotic plants and variety of *Bradyrhizobium* spp. associated with *Cytisus proliferus*.

Ochrobactrum is classified in the family Brucellaceae in the order of Rhizobiales (Garrity et al., 2004). *Rhizobium* belonging to the family of Rhizobiaceae, and *Mesorhizobium* and *Bradyrhizobium* belonging to Phyllobacteriaceae and Bradyrhizobiaceae, respectively, are also in Rhizobiales (Table 3.1) (Garrity et al., 2004). Not all the families from this order of Rhizobiales can fix nitrogen with their symbiotic plants, but so far, at least six genera of N-fixing bacteria (such as *Bradyrhizobium*, *Devosia*, *Mesorhizobium* and *Rhizobium*) have been idenrified from the families of Bradyrhizobiaceae, Hyphomicrobiaceae, Phyllobacteriaceae, and Rhizobiaceae (Beck et al., 2015; Carvalho et al., 2010; Gupta, 2005).

One strain as a novel N-fixing species was isolated and identified as *Ochrobactrum* sp. from *Acacia mangium* grown in Philippines (Ngom et al., 2004). Ngom et al. (2004) reported that the strain could fix nitrogen as well as other rhizobium strains which were also isolated from this species. Strains isolated from root nodules of *Lupinus honoratus* in Argentina were reported belong to a new species of *Ochrobactrum*, which was able to re-nodulate *Lupinus albus* (Trujillo et al., 2005) and was named *Ochrobactrum lupini* sp. nov. Trujillo et al. (2005). *Ochrobactrum cytisi* sp. nov. (a novel species from the work of Zurdo-Pineiro Zurdo-Pineiro et al. (2007)) was isolated from *Cytisus scoparius* in Spain. The *nodD* and *nifH* genes of that strain were related to those of rhizobial species associated with *Phaseolus*, *Leucaena*, *Trifolium* and *Lupinus* (Zurdo-Pineiro et al., 2007).

In the present study, four isolates were obtained from *Astragalus cicer* and *Cytisus proliferus* root nodules and identified as *Ochrobactrum* spp. by 16S rRNA analyses. Also, *recA* gene analysis showed they all belong to *Ochrobactrum* spp.. In contrast, the *nifH* gene phylogenetic results showed they were located in *Mesorhizobium* (*Astragalus cicer*) and *Bradyrhizobium* (*Cytisus proliferus*) braches. The findings from this work are similar to the previous findings which the *Ochrobactrum* spp. isolated from different plants contained N-fixing gene (*nifH*) and they were all related to rhizobia. This indicates that the nitrogen fixation gene of the *Ochrobactrum spp.* was not unique but depends on the rhizobia which associated with the hosts. The nitrogen fixation gene may not initially come

from the *Ochrobactrum spp.;* it may transferred from the rhizobia or else it may be induced through infection of their hosts. In the recent decade, some researches demonstrated that *Ochrobactrum* spp. may have N-fixing capability. However, no report has been found of *Ochrobactrum* spp. isolated from *Astragalus cicer* and *Cytisus proliferus*. In this study, all the four *Ochrobactrum* spp. from the two N-fixing species contain *nifH* gene and could form nodules with their hosts.

Table 3.1 Classification of bacteria isolated from exotic N-fixers (Garrity et al., 2004).

Class	Alphaproteobacteria		
Order	Rhizobiales		
Family	Phyllobacteriaceae	Bradyrhizobiaceae	Brucellaceae
Genus	*Mesorhizobium*	*Bradyrhizobium*	*Ochrobactrum*
Host in this study	*Securigera varia* *Astragalus cicer*	*Cytisus proliferus*	*Astragalus cicer* *Cytisus proliferus*

3.5 Conclusions

Work reported in this chapter successfully isolated, identified and re-inoculated N-fixing bacteria from the three species of exotic plants. The results have identified that significant diversity exists between the actual symbionts associated with the plants. Clearly there was also some commonality between the symbionts associated with the three plant species, and with plant-rhizobia associations reported in the literature. In total, thirteen isolates were obtained in this work including six *Mesorhizobium* spp. isolated from *Securigera varia* and *Astragalus cicer,* three *Bradyrhizobium* spp. from *Cytisus proliferus* and four *Ochrobactrum* spp. from *Astragalus cicer* and *Cytisus proliferus*. *Mesorhizobium* spp. isolated from *Securigera varia* in this work were most closely related to *M. caraganae* (in terms of 16S rRNA gene). Two of the isolates from *Astragalus cicer* that were identified as *Mesorhizobium* spp. were closely related to *M. tianshanense* (in terms of 16S rRNA gene). *Bradyrhizobium* spp. isolated from *Cytisus proliferus* were similar to a *Bradyrhizobium cytisus* strain (in terms of 16S rRNA gene) previously isolated from *Cytisus* in New Zealand. *Ochrobactrum* spp. isolated from *Astragalus cicer* and *Cytisus proliferus* root nodules, and they all contained *nifH* genes (the gene participate encoding enzymes which are involved in N fixation). These *Ochrobactrum* spp. were able to form nodules with their symbiotic plants (*Astragalus cicer* and *Cytisus proliferus*).

Chapter 4

The influence of N-fixing bacteria on growth of legumes

4.1 Introduction

Biological nitrogen fixation only occurs in nature when mediated by bacteria and legumes respond to rhizobia infection by developing nodules (Burns & Hardy, 2012). Both naturally established and introduced bacteria associated with root nodules are able to improve legume productivity and consequentially benefit soil fertility (Brockwell et al., 1995). Most of legumes and other N-fixing species in association with specific rhizobia for maximizing the functional nitrogen fixation (Bontemps et al., 2010; Sarig et al., 1986). N-fixing strains which associate with one species could fix different amount of nitrogen depend on their effective rate (Bever & Simms, 2000; Mahdi et al., 2010). Nitrogen fixation rate is directly related to legume plant growth rate. Any factors which could reduce plant growth such as drought, insufficient sunlight, low temperature, and limited plant nutrients will also reduce nitrogen fixation (Streeter, 2003; Weisz et al., 1985).

Catroux et al. (2001) discussed the use of rhizobia inoculants to increase nitrogen fixation and yield of legume crops. Rhizobia can not only be used to their associated species of legume, but some strains also promote growth and increase yield in non-legume plants such as rice (Biswas et al., 2000; Santi et al., 2013). The associated bacterial symbiont also benefits from the relationship. The plant provides energy to the bacteria from photosynthates and other nutrients (Beattie, 2007; Burris & Roberts, 1993). Nitrogen fixation mechanisms differ between plant species. Common beans generally fix less nitrogen than they require, whereas some grain legumes like soybean and peanuts have the capability to fix more nitrogen than they need (Lindemann & Glover, 2003).

Thirteen isolates were taken from three species of exotic legumes in the study reported in the previous chapter (Chapter 3). All the isolates contained the *nifH* gene which relates to the process of nitrogen fixation. The same isolates were used in the present chapter in order to investigate whether the isolated N-fixing related bacteria could influence the growth of their hosts. Additional isolates taken from native legumes in an earlier study by Tan (2014) were also used in this work to provide a comparison of different inoculants, especially between native and exotic legumes.

Legumes are known to fix atmospheric nitrogen to sustain their own growth, and it is unlikely that the same symbionts benefit plants from other families. However, this was tested in the present study by including *Pomaderris amoena* (Rhamnaceae), a native shrub present in the Eyrewell study

site of particular conservation significance. This species establishes quickly from seed stores following soil disruption and fire and is may have a role in restoration management (Dollery, 2017). However, little research has been carried out on this native species. The question was raised as to whether native or exotic legumes could affect the growth of this native non N-fixing shrub. To address the question above, *Pomaderris amoena* was planted with native and exotic legume species in a greenhouse experiment.

The aim of this chapter was to investigate the influence of N-fixing bacteria on the growth of native and exotic legumes and the subsequent effects of legumes on non N-fixing plants, using pot experiments in the glasshouse. Experiment I focused on the effects of inoculation on the growth of the host plants, and the significance of soil nitrogen status. Experiment II investigated the impacts of native and exotic legumes on the growth of the non N-fixing native plant (*Pomaderris amoena*).

4.2 Experiment I (The influence of related N-fixing bacteria on growth of native and exotic legumes)

4.2.1 Materials and methods

4.2.1.1 Plants and soil

Three native species (*Sophora microphylla*, *Sophora prostrata*, *Carmichaelia australis*) and 3 exotic species (*Securigera varia*, *Cytisus proliferus*, *Astragalus cicer*) were used in this experiment. Native plants were purchased from Motukarara Department of Conservation Nursery, Canterbury. All the native seedlings were approximately one-year old. The exotic species were three-months old and were grown from seed by the author. The source of exotic seeds is detailed in Chapter 3 (#3.2.1). Soil was collected from a restoration area in Eyrewell, Canterbury (172.316°, -43.451°) to a depth of 0-15 cm, and sterilized in an autoclave at 121 °C for an hour prior to use. Plant roots were washed and soaked in 0.25g sodium hypochlorite solution for 10 to 15 seconds then rinsed with sterilized water. Pots were soaked into 2% sodium hypochlorite solution for 15 seconds then air-dried overnight.

4.2.1.2 Microbial assay

Rhizobium strains of native legumes (*Sophora microphylla*, *Sophora prostrata* and *Carmichaelia australis*) had been isolated in a recent previous study (Tan, 2014) and the cultures had been maintained at Lincoln University. Strains of exotic species including rhizobia and *Ochrobactrum* spp. were isolated from the present research project (Chapter 3). Strain number and 16S rRNA access

number of NCBI are presented in Table 4.1. Strains were mix cultured (allied to host species) in 200ml YMB media (Table A.1) at 25 °C for 2-4 days for inoculation (approximately $4*10^8$ cfu ml^{-1}).

Table 4.1 Inoculation strains isolated from native and exotic species and their access number of 16S rRNA (NCBI).

Species (native)	Strain		16S rRNA	Species (exotic)	Strain		16S rRNA
Carmichaelia australis	ICMP 19041	*Mesorhizobium*	JQ963060	*Securigera varia*	C1	*Mesorhizobium*	KX770727
	ICMP 13190	*Mesorhizobium*	AY491071		C2	*Mesorhizobium*	KX770728
	ICMP 19419	*Mesorhizobium*	JQ963062		C3	*Mesorhizobium*	KX770729
					C4	*Mesorhizobium*	KX770730
Sophora prostrata	ICMP 19545	*Mesorhizobium*	KC237410	*Atragalus cicer*	A1	*Mesorhizobium*	KX770731
	ICMP19547	*Mesorhizobium*	KC237430		A3	*Mesorhizobium*	KX770732
	ICMP 19542	*Mesorhizobium*	KC237412		A2	*Ochrobactrum*	KX770733
	ICMP 19543	*Mesorhizobium*	KC237431		A5	*Ochrobactrum*	KX770734
Sophora microphylla	ICMP19512	*Mesorhizobium*	KC237394	*Cytisus proliferus*	T8	*Mesorhizobium*	KX770736
	ICMP 19517	*Mesorhizobium*	KC237399		T9	*Mesorhizobium*	KX770737
	ICMP 19524	*Mesorhizobium*	KC237414		T10	*Mesorhizobium*	KX770738
	ICMP 19535	*Mesorhizobium*	KC237424		T7	*Ochrobactrum*	KX770735
					T12	*Ochrobactrum*	KX770739

4.2.1.3 Experiment design and plant maintenance

The six plant species (3 native and 3 exotic) were each divided into a control group (without inoculation) and a test group (with inoculation), with 4 replicates of each. A total of 30ml of each culture mix (prepared as in #4.2.1.2) was inoculated on to the surface of the soil in pots containing plants from the test group once planted. The control group plants were inoculated with 30ml YMB media without any bacteria. All plants were grown in a Lincoln University greenhouse for 6 weeks with average temperature of 25°C in daytime and 15°C at night.

4.2.1.4 Measurements

Plants were harvested after six weeks growth and the dry biomass of above ground shoots and below ground roots were measured after being dried at 60°C for 3-5 days. The number of nodules was counted and the soil was analyzed for pH, total nitrogen and carbon, inorganic nitrogen (NH_4^+ and NO_3^-) in both the control and test groups.

NH_4^+ and NO_3^- concentrations in the soil were determined following extraction with KCl. Four grams of fresh soil from each pot were transferred to 50 ml tubes, adding 40ml of 2 M KCl, then shaking for

1 hour, centrifuged at 2,000 rpm (10 mins) and filtered (Whatman 41 filter paper) following the procedure described Clough et al. (2001). All samples were analyzed by Flow Injection Analysis (FIA, Foss FIAstar 5000 triple channel, Foss Tecator, Sweden). The remaining soil was air dried for 48 to 72 hours, then ground and sieved (< 2 mm) for soil pH and total nitrogen and carbon analysis. Soil pH was measured following suspension of 5 g of dry soil in deionized water for 4 hours at the ratio of 1:5 of soil: solution (S20 SevenEasy^{TM} pH meter, Mettler-Toledo, Switzerland). Total nitrogen and carbon were analyzed by Vario-Max CN elemental analyser (Elementar GmbH, Germany) at Lincoln University.

4.2.1.5 Statistic analysis

Analysis of plant biomass, soil ammonium, soil nitrate, soil pH and total soil N/C was carried out using ANOVA (one-way) Fisher's comparisons (Minitab, version 17) to investigate the differences between the test and control group.

4.2.2 Results

4.2.2.1 Plant biomass and nodulation

For all the native species, there was no significant difference between the inoculated and non-inoculated groups in terms of total plant dry biomass. However, root dry biomass of *S. microphylla* and *S. prostrata* was higher than *C. australis* with inoculation. For the exotic species, *C. proliferus* had higher biomass on both shoot and root with inoculation application than without (Figure 4.1, $P<0.05$). For *A. cicer* and *C. varia*, there was no significant difference in plant dry weight between the control and the test group.

The two exotic species (*A. cicer* and *C. proliferus*) had more nodules with inoculation than without (Figure 4.2, $p<0.05$). Otherwise, there was no difference in the number of nodules in all the native species and exotic species of *S. varia*.

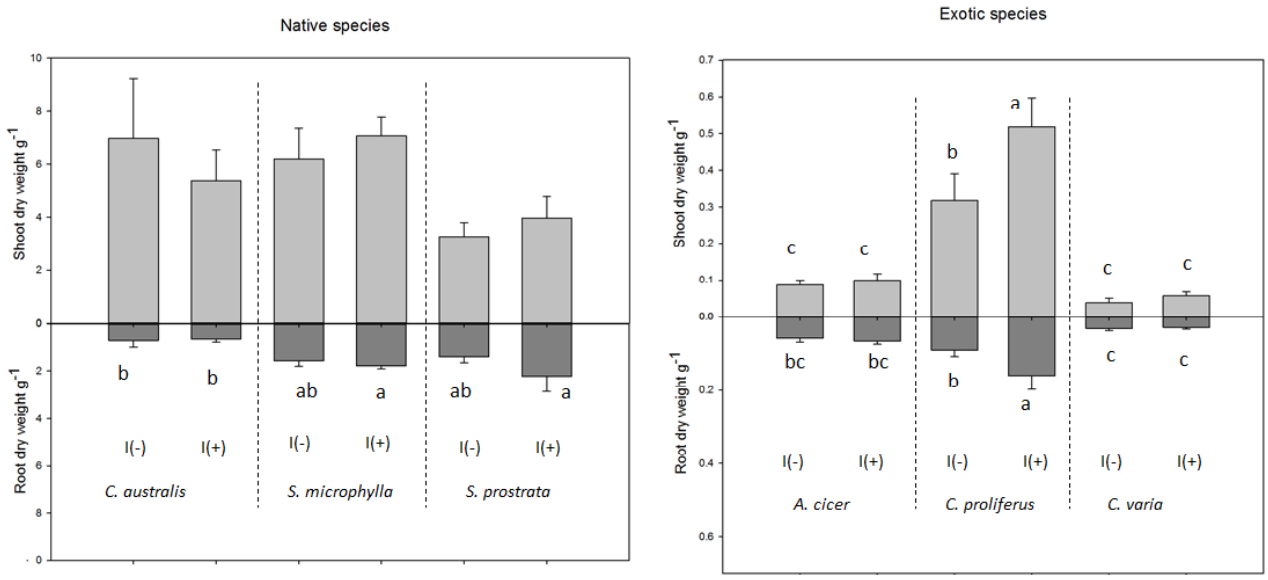

Figure 4.1 Mean (±SE) dry weight (shoot/root) of the native and exotic N-fixing species. Results were tested using ANOVA (one-way) Fisher's comparisons, replicate n=4, p<0.05 (shoot dry weight of native species p>0.05).

4.2.2.2 Soil properties

At the end of the experimental period, soil mineral nitrogen (NO_3^- and NH_4^+) and soil pH differed significantly with and without inoculation, in a native N-fixer (*S. prostrata*). Total soil nitrogen and carbon were also analyses but there was no significant difference between the control and inoculation groups for any species (data not shown).

Soil NH_4^+ content with *S. prostrata* had a positive response to inoculation (Figure 4.3, $p<0.05$) whereas the other species did not. *Sophora prostrata* provided more soil NH_4^+ with inoculation application than *C. australis* and all the exotic species ($p<0.05$). Without inoculation, soil containing *S. microphylla* had the highest soil NH_4^+ content than other native and exotic species (Figure 4.3, $p<0.05$).

In terms of soil NO_3^-, the soil of *S. prostrata* with inoculation was higher than without (Figure 4.3, $p<0.05$). There was no significant difference for the other species between the control and the test group. Without inoculation, the soil NO_3^- content of *S. prostrata, C. varia and A. cicer* were higher than *C. australis* ($p<0.05$). With inoculation, the soil NO_3^- content of *S. prostrata* was the highest among all species ($p<0.05$).

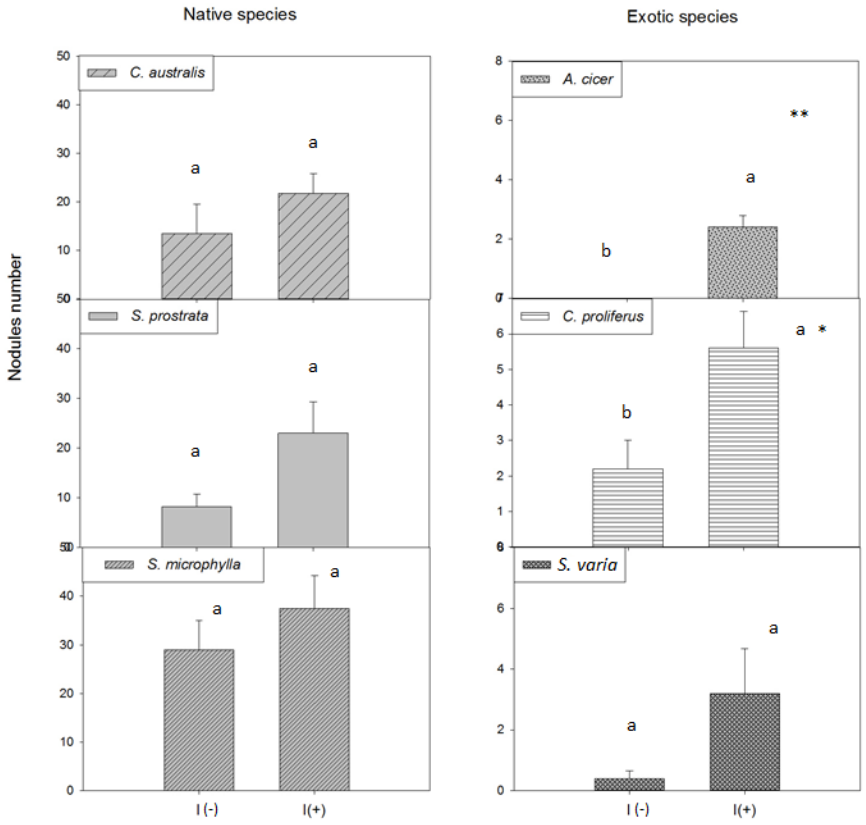

Figure 4.2 Mean (±SE) number of nodules from the native and exotic N-fixing species. Results were tested using ANOVA (one-way) Fisher's comparisons, n=4. *, ** indicates the effect being significant at p<0.05, p<0.01.

Soil pH

Soil pH of *S. prostrata* with inoculation was higher than when no inoculants had been added (Figure 4.4, p<0.05). There was no significant difference between the control group and the inoculation group for the other species. Apart from *S. microphlylla*, soil pH with all the species appeared to be increased with inoculation, but not significantly. With inoculation, soil pH with *S. microphylla* was lower than with other species (p<0.05).

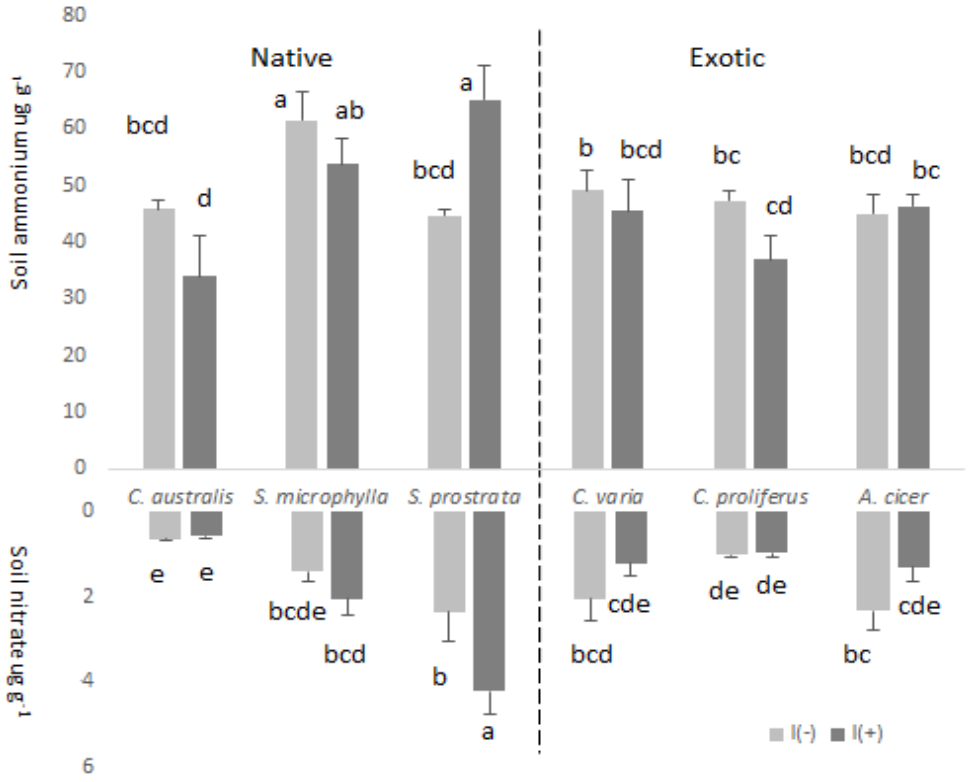

Figure 4.3 Mean (±SE) ammonium and nitrate content of the native and exotic species with (I (+)) and without inoculation (I (-)). Soil pH was tested using ANOVA (one-way) Fisher's comparisons, n=4, p<0.05. Histogram bars which share letters are not significantly different in native and exotic species.

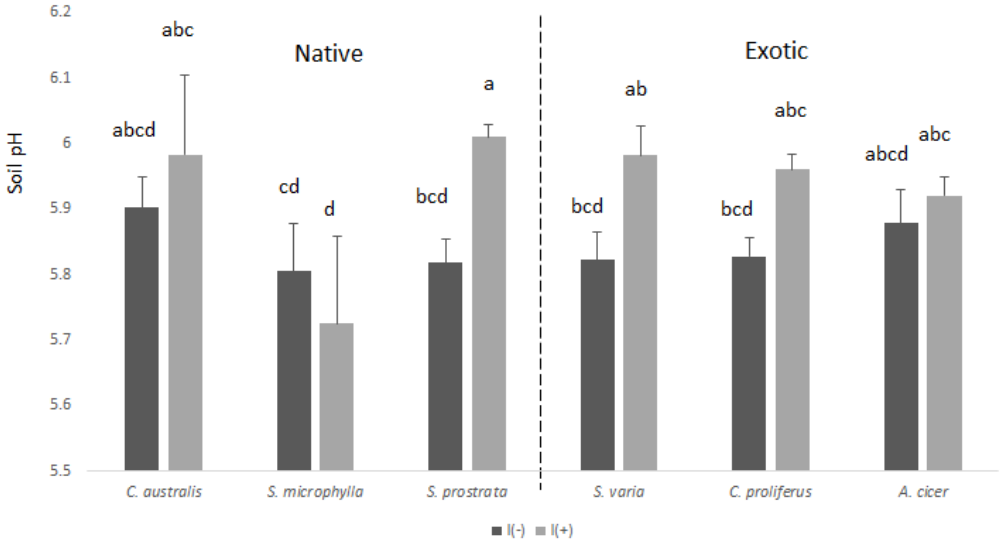

Figure 4.4 Mean (±SE) soil pH in pots with native and exotic species with (I (+)) and without inoculation (I (-)). The soil pH was tested using ANOVA (one-way) Fisher's comparisons, n=4, p<0.05. Histogram bars which share letters are not significantly different in native and exotic species.

4.3 Experiment II (The effects of N-fixers on *Pomaderris amoena*)

4.3.1 Materials and methods

4.3.1.1 Seed and soil

In this experiment two native N-fixing species (*S. microphylla* and *C. australis*) and two exotic species (*S. varia* and *C. proliferus*) were grown together with *P. amoena* in pots. Seeds of *C. australis* and *P. amoena* were collected (by Dollery, 2015) from Eyrewell forest, Canterbury (-43.434, 172.295). Seeds were harvested from multiple plants in the field that were about 1.5m (*C. australis*) and 0.5m (*P. amoena*) tall. *Sophora microphylla* seeds were collected (by the author, 2015) from multiple trees (2-3 meters) at Mount Grand (-44.66, 169.33). Mount Grand is located on the east of Clutha Valley Basin in Central Otago in the South Island of New Zealand. The source of *S. varia* and *C. proliferus* seeds is detailed in Chapter 3 (#3.2.1). Soil was collected from the Eyrewell area referred to in #4.2.1.

4.3.1.2 Experiment design and plant maintance

Seeds of N-fixing species (except *C. australis*) were scarified using a file and soaked in warm water overnight. Twelve pots containing 3 replicates of 2 native and 2 exotic N-fixing species. Each pot containing ten seeds were sown for each of the species. The *C. australis* seeds were sown into the pots directly without any preparation process. After 4 weeks growth, ten of *P. amoena* seeds were soaked in hot water overnight and sown into each pot of the native and exotic legumes. Twenty of the *P. amoena* seeds were sown into 3 pots without any legumes as a reference (control) group. All seedlings were grown in a greenhouse at Lincoln University nursery for 3 months, with an average temperature of 25°C during the day and 15°C at night.

4.3.1.3 Measurements

Plant dry biomass (whole plants) and height (shoot and root) were measured after 3 months' growth. Roots were carefully separated from the soil following harvest and numbers of nodules were counted. Soil pH, inorganic nitrogen (NH_4^+ and NO_3^-) and soil total N and C were analyzed as described previously Section 4.2.1.4.

4.3.1.4 Statistic analysis

Analysis of plant dry weight, height, soil mineral nitrogen, soil pH and total soil N and C were tested by ANOVA (one-way) Fisher's comparisons (Minitab, version 17).

4.3.2 Results

4.3.2.1 *Pomaderris amoena* germination rates in different plant groups

The percentage of germination rates of *P. amoena* ranged from 27% to 47% within the legume species. The percentage of germination rate within the control group (*P. amoena* only) is 32%. The rates of *P. amoena* with the exotic legume species were 33% (*S. varia*) and 43% (*C. proliferus*) and with native species were 27% (*S. microphylla*) and 47% (*C. australis*).

4.3.2.2 Plant biomass of *P. amoena*

There were some significant differences in plant biomass of *P. amoena* when grown with different native and exotic legume species. *Pomaderris amoena* had higher dry biomass when grown with the native legume plants (*C. australis*), compared to with exotic species (*S. varia* and *C. proliferus*) and the control (Figure 4.5a, $P<0.05$).

The height attained by *P. amoena* was significantly different when grown with native and exotic legumes (Figure 4.5b, $p<0.05$). In *P. amoena* length of shoots and roots were higher when grown with the two native species than with the two exotic species ($p<0.05$). There was no significant difference in *P. amoena* height between the control and the *P. amoena* grown with the two exotic N-fixing species (Figure 4.5b). Figure 4.6 showed the comparison of the growth of *P. amoena* with native and exotic legumes in pots.

4.3.2.3 Soil properties affected by different plants

There were significant differences in soil NH_4^+, soil total nitrogen (N%), total carbon (C%) and soil C:N ratio between the control (which contained only *P. amoena*) and *P. amoena* with native or exotic legumes (Table 4.2, $p<0.05$). There was no significant difference in soil NO_3^- content and soil pH between the treatments (Table 4.2).

P. amoena grown with *S.varia* provided higher soil NH_4^+ than the control, *P. amoena* with *C. australis*, and *P. amoena* with *C. proliferus* ($p<0.05$). *P. amoena* with *C. proliferus* had higher soil N and C than the control and with *C. australis* ($p<0.01$). *P. amoena* with *C. australis* had the lowest soil N rate among all the plant groups ($p<0.01$). The control showed the lowest soil total C rate in all the plant groups ($P<0.01$). There was no significant difference for soil NH_4^+, total C and C:N ratio between *P. amoena* grown with the two native species (*C. australis* and *S. microphylla*). The C:N ratio of *P. amoena* grown with *C. australis* was higher than the control and *P. amoena* grown with *S. varia* ($P<0.05$).

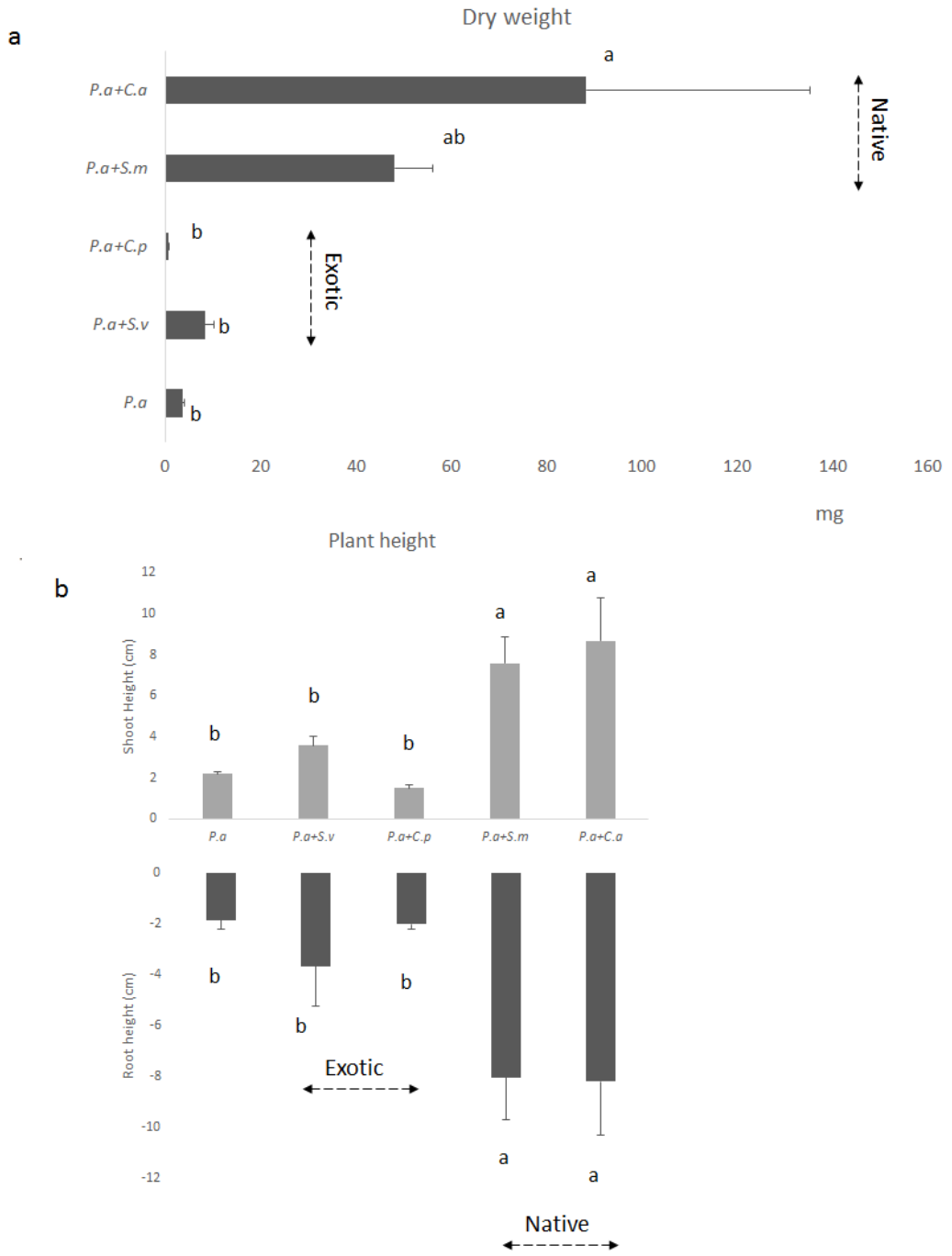

Figure 4.5 Mean (±SE) plant biomass and height of *P. amoena* and *P. amoena* with the growth of different native and exotic N-fixers after 3 months growth period. Results were tested using ANOVA (one-way) Fisher's comparisons, n=5, p<0.05. Bars do not share letters are significantly different in dry weight, shoot and root height respectively. *P.a= Pomaderris amoena*, *S.v= Securigera varia, C.p= Cytisus proliferus, S.m= Sophora Microphylla, C.a= Carmichaelia australis.*

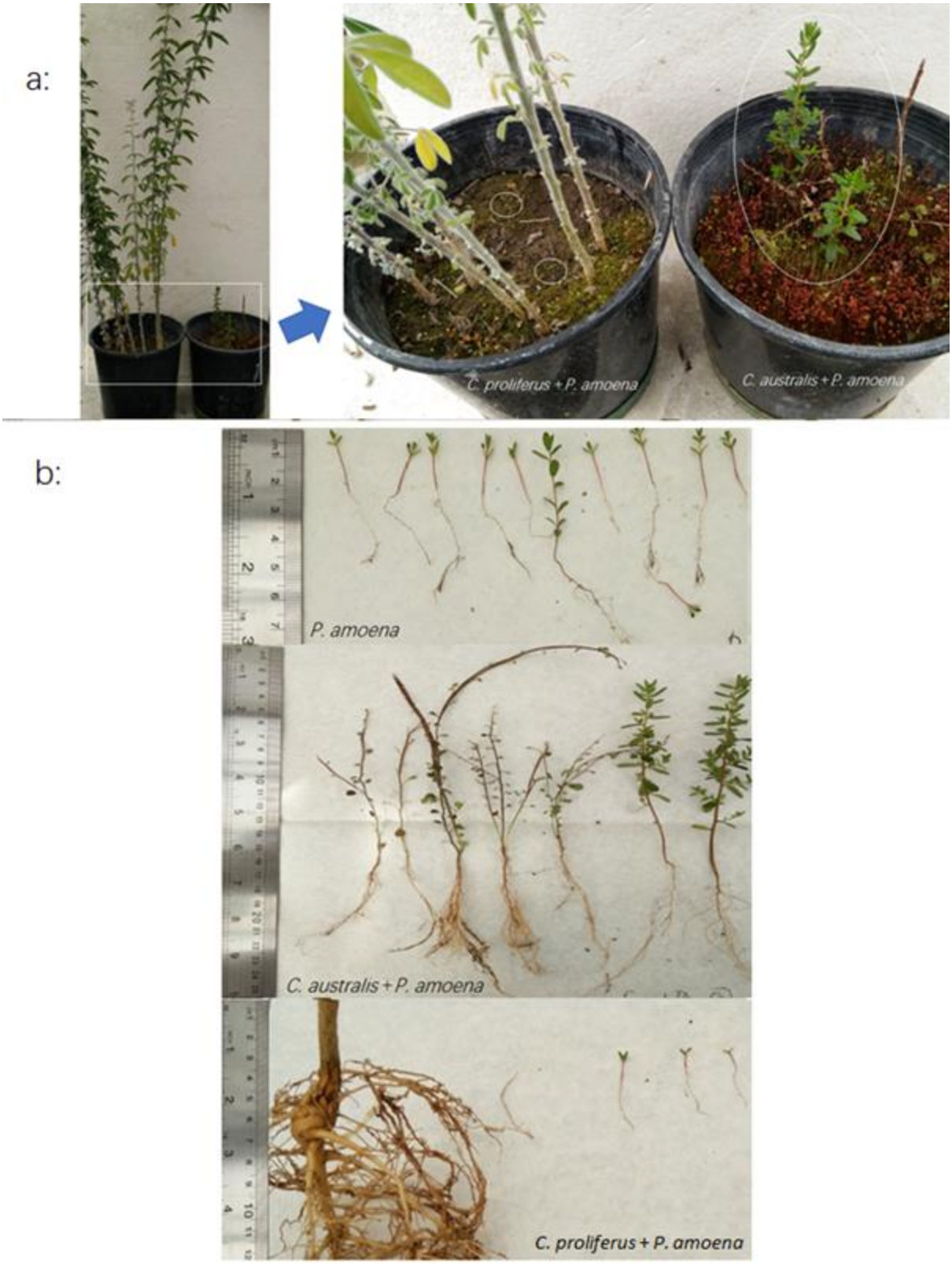

Figure 4.6 a: The growth of *P. amoena* with the native and exotic species (*C. australis* and *C. proliferus*). Seedlings of *P. amoena* shown in white circle. The seedlings of *P. amoena* were really small when it grown with *C. proliferus* and it is difficult to see in the picture; b: The size of *P. amoena* in the control, and the *P. amoena* with the native (*C. australis*) and exotic plants (*C. proliferus*).

Table 4.2 Effects of the growth of *P. amoena*, and it grown with the different N-fixing species on soil property. Results were tested using ANOVA (one-way) Fisher's comparison (n=3). Numbers in brackets indicate the standard error of the mean (SE). Numbers which share letters are not significantly different in each column. *P.a= Pomaderris amoena*, *S.v= Securigera varia, C.p= Cytisus proliferus, S.m= Sophora Microphylla, C.a= Carmichaelia australis.*

Species	Soil property					
	NH_4^+(ug g^{-1})	NO_3^-(ug g^{-1})	pH	N (%)	C (%)	CN Ratio
P. amoena	11.82 (11.08)^{b}	0.73 (0.10)^{b}	6.21 (0.04) ^{a}	0.193 (0.004) ^{b}	2.64 (0.06) ^{c}	13.73 (0.12)^{c}
P. a +S. m	22.29 (1.07)^{ab}	0.85 (0.02) ^{ab}	6.11 (0.04) ^{a}	0.197 (0.002) ^{ab}	3.00 (0.07) ^{ab}	15.22 (0.28) ^{ab}
P. a +C. a	4.80 (2.02) ^{b}	0.85 (0.07) ^{ab}	6.47 (0.19) ^{a}	0.181 (0.004)^{c}	2.87 (0.07) ^{b}	15.94 (0.73) ^{a}
P. a +S. v	38.2 (4.69)^{a}	0.97 (0.01)^{a}	6.08 (0.02) ^{a}	0.197 (0.002) ^{ab}	2.87 (0.03) ^{b}	14.63 (0.28)^{bc}
P. a +C. p	11.76 (8.81) ^{b}	0.83 (0.02) ^{ab}	6.39 (0.22) ^{a}	0.202 (0.002) ^{a}	3.12 (0.05) ^{a}	15.41 (0.17) ^{ab}
P value	**<0.05**	0.098	0.232	**<0.01**	**<0.01**	**<0.05**

4.4 Discussion

4.4.1 Experiment I

Plant biomass and nodulation

Rhizobia are widely known to provide significant amounts of nitrogen via their symbiotic relationship that enhance plant yield, from studies elsewhere (Park et al., 2010). In this study, however, inoculants did not make any difference to biomass and nodulation of the three native nitrogen-fixing plants (*C. australis*, *S. microphylla* and *S. prostrata*; one-year old) after 6 weeks growth. In an earlier study, Tan (2014) inoculated *S. microphylla* seedlings, with rhizobia; after 14 weeks, he found the total dry weight of plants was higher with inoculation than without. Pérez-Fernández and Lamont (2003) studied 12 different legume seedlings from Spain and Australia, and it showed inoculation was effective in increasing the biomass of all the species after 6 months growth from seeds. The different results in the present study may be due to the differences of plant ages, or else to differences in the plant growth period. Additionally, the efficiency of the bacteria may be considered as another reason that whether they are effective or not after inoculation. It is also possible that inoculants may be more effective to the early stage of nodulation than after nodules have already formed, or more effective after a long growth period rather than a short growth period for the plants.

Exotic legume species were more responsive to inoculants than native species in the present study. The biomass of *C. proliferus* and nodulation of *A. cicer* and *C. proliferus* were greater with inoculation

than without. Liu (2014) reported that *Cytisus palmensis, Cytisus scoparius* and *Ulex europaeus* (a common exotic legume in New Zealand) responded positively to the inoculants previously isolated from their hosts. In the present study, the more effective inoculation of exotic legumes than native species may be also due to the different plant ages. The exotic legumes were three-months old, while native legumes were one-year old. Inoculants may be more effective on small plants during the short growth period. Additionally, *Cytisus proliferus* is faster growing than other native legumes and so may show the effects of inoculation more readily.

Soil modification by inoculation

Inoculation of *S. prostrata* enhanced soil mineral N in soil ammonium and nitrate, with no significant difference for other legume species. Inoculation significantly increased soil pH with *S. prostrata*, while other species did not. The efficiency of rhizobia and nodulation are affected by soil pH (Drew et al., 2012; Ullah, 2010). Different legume-rhizobia symbioses are able to tolerate different soil pH levels (Drew et al., 2012). El-Kherbawy et al. (1989) reported that alfalfa was not able to survive at soil pH of 4.3 and 5.3, while plant growth was significantly increased when inoculated with rhizobia at soil pH of 6.0 to 7.2. Soil pH ranged from 5.7 to 6.0 in this study. Interestingly, inoculants increased *S. prostrata* soil pH, and the higher soil pH may have enabled the rhizobia and *S. prostrata* symbiosis to release more soil mineral nitrogen into the soil. No studies were found to support the findings that rhizobia are able to increase soil pH. However it could be suggested that rhizobia may be able to increase soil pH due to the nitrogen fixation reaction requiring hydrogen ions, the formula is shown below:

$N_2 + 8H^+ + 8e^- + 16ATP \rightarrow 2NH_3 + H_2 + 16ADP + 16Pi$ (Halbleib & Ludden, 2000).

Hydrogen ions are required through nitrogen fixation, and so decreasing soil hydrogen ions leads to an increased pH. Although no significant difference in soil pH was found with other species, the average soil pH was higher with inoculation than without (except *S. microphylla*). Therefore, inoculation and nitrogen fixation could enhance soil pH values, but the effects vary with different N-fixing species. N-fixing bacteria may have different efficiency for nitrogen fixation dependent on the strain species and soil pH. Additionally, many New Zealand soils are naturally acidic and therefore it is possible that rhizobia exist in New Zealand are adapted to more acidic conditions (Sparling & Schipper, 2002; Sparling & Schipper, 2004).

4.4.2 Experiment II

The growth of *P. amoena* with legumes and soil status

Native legume plants increased the growth of *P. amoena* (a non N-fixing plant) in terms of dry weight and height, suggesting that *P. amoena* probably received some nitrogen from the legumes. Prior studies indicate that nitrogen could transfer from forage legumes (alfalfa, red clover and trefoil) to grass. The amount of transferred nitrogen depends on distance of transfer and ratio bewteen legumes and grasses (Brophy et al., 1987; Heichel & Henjum, 1991; Ta & Faris, 1987). Characteristics of roots may also affect the nitrogen transference from legumes to their neighbouring plants (Pirhofer-Walzl et al., 2012). Legumes may also enable neighbouring plants to receive more nutrients (P and Mn) which are produced or made available by legumes (Gardner & Boundy, 1983). *Carmichaelia australis* grown with *P. amoena* produced the highest soil C:N ratio (Table 4.2), and this combination of planting may provide more optimal conditions for soil microbes to release more nutrients due to higher soil C:N ratios are more suitable for microorganisms (Carroll & Weigle, 2016; Thompson, 1952) and so promoted the growth of *P. amoena*. It could be speculated that this may simultaneously be beneficial in terms of limiting nitrous oxide emission by reduced nitrification of denitrification associated with increasing C:N ratio (Huang et al., 2004; Klemedtsson et al., 2005). The other three legume species also increased C:N ratio with *P. amoena* compared to the control, further indicating that legume species may reduce nitrous oxide emission.

The growth of *P. amoena* did not benefit from exotic legume species in this experiment, maybe due to the competition between plants. The dry biomass of *C. proliferus* was much greater than *P. amoena*, and also the other legume species (Figure 4.7). *Cytisus proliferus* took up much more pot space and probably absorbed more soil nutrients than *P. amoena*. This size-symmetry competition means that plants take up resources in proportion to their size (Schwinning & Weiner, 1998). There would also be increased competition for light and water between plants. In this study, the biomass of *P. amoena* grown with *C. proliferus* was no less than the control (*P. amoena* without any of the N-fixers). Therefore, although the exotic species (*C. proliferus*) did not promote the growth of *P. amoena*, it also did not inhibit the growth. Fargione and Tilman (2006) studied different grassland species within different communities, and they found plant species had high biomass, and also produced high root length density and reduced soil N contents. In the present study, the results differed to the grassland species, *Cytisus profliferus* had a much higher biomass with the growth of *P. amoena* but did not reduce soil N content compared to the control group. *Pomaderris amoena* grown with legume species produced a higher total soil carbon than the control. This maybe because more organisms are contained by legume-rhizobia symbiosis due to organisms and dead plant

tissues contribute most of the total soil N and C (Bååth et al., 1978) while inorganic forms are much less than organic forms in soil (Macdonald et al., 1989; Nasholm et al., 1998; Nelson & Sommers, 1996; Torn et al., 1997).

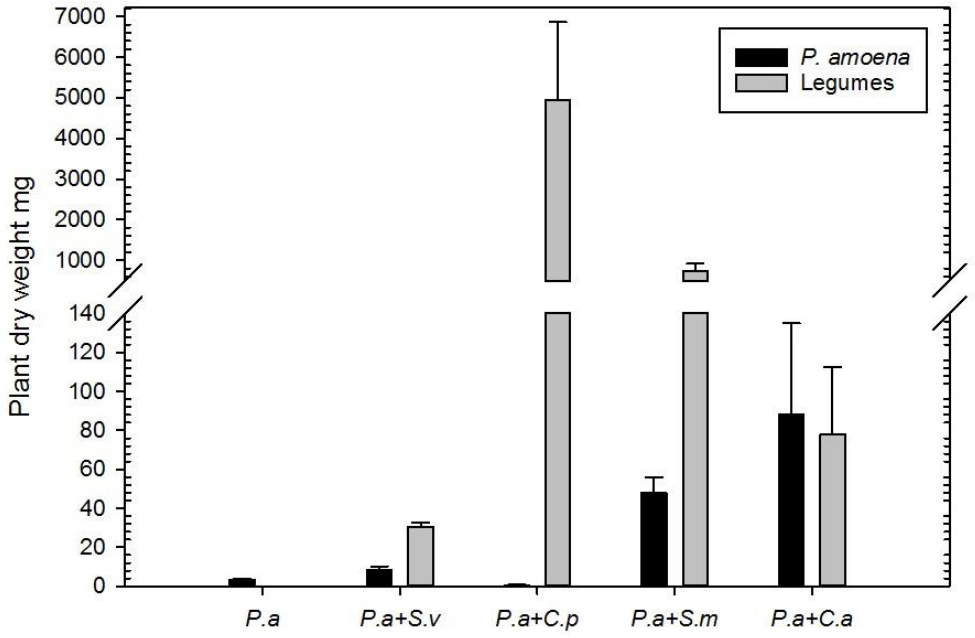

Figure 4.7 Mean (±SE) plant dry weight of *P. amoena* (*P.a*), and it grown with exotic legumes (*S.v*=*S. varia*, *C.p*=*C. proliferus*) and native legumes (*S.*m=*S. microphylla*, *C.a*=*C. australis*).

Sophora microphylla and *C. australis* developed more biomass than *S. varia* (Figure 4.7). According to size-symmetry competition, *P. amoena* should uptake more nutrients when grown with *S. varia* than with the two native legumes, but the results showed the opposite effect. This may be due to plant initially take up nitrate from in the soil, secondarily ammonium convert to nitrate for continuously use (Foth & Ellis, 1997). There was no significant difference in soil nitrate levels between the plant groups, but *P. amoena* grown with *S. varia* had more soil ammonium than with the native species (Table 4.2). Therefore, there was a less soil ammonium converted to nitrate for the group of *P. amoena* with *S. varia* than the other groups, which may have led to less available nitrogen in the soil for the growth of *P. amoena*.

The significance of moss growth in pots

Moss grew prolifically with some of the plant seedlings. Within a few months moss dominated in the pots of *P. amoena* (picture not shown), and plant groups of *P. amoena* + *C. australis*, *P. amoena* + *S. microphylla* and *P. amoena* + *S. varia* (Figure 4.8). However, little moss growth occurred in the pots

when *P. amoena* grown with *C. proliferus* (Figure 4.8). The plants were watered regularly in similar amounts, but *C. proliferus* grew much faster than the other plants, absorbing more water and leading to a lower soil moisture content (Figure 4.9). Moss reproduction critically relies on moisture content as mosses require water for fertilization (Bell & Bliss, 1980; Hallingbäck & Hodgetts, 2000). This probably led to the different performance of mosses within the different plant groups.

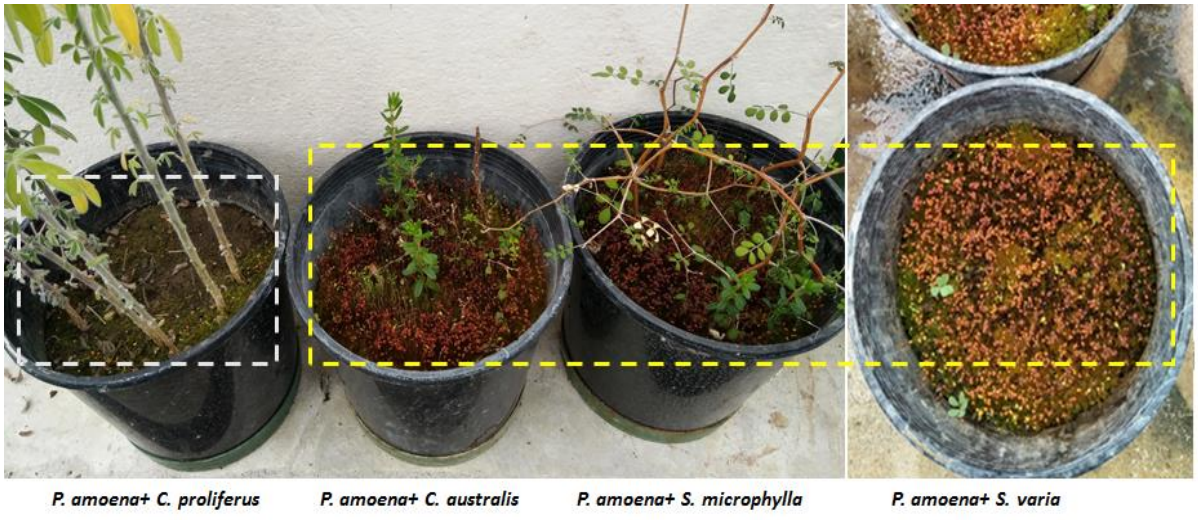

Figure 4.8 Moss growth status in the pots with different plant groups.

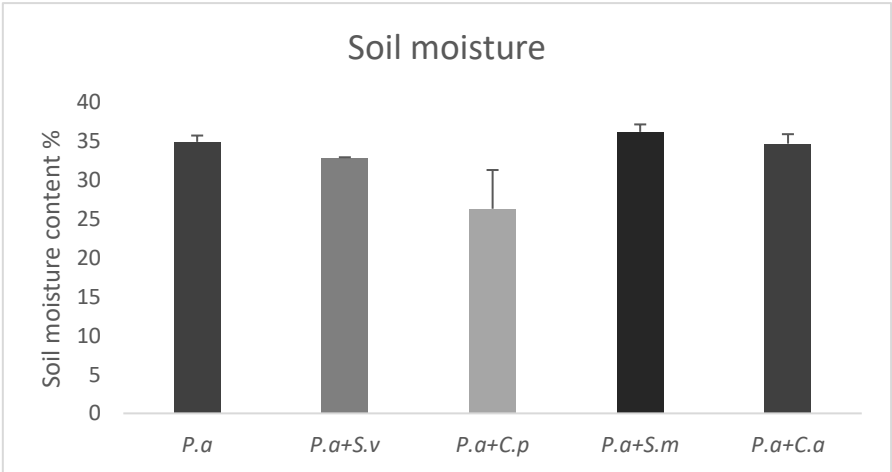

Figure 4.9 Soil moisture contents of *P. amoena*, and *P. amoena* with native and exotic legume species. Results were tested using ANOVA (one-way) Fisher's comparisons (n=3, p<0.05). Bars which share letters are not significantly different. *P.a= Pomaderris amoena*, *S.v= Securigera varia, C.p= Cytisus proliferus, S.m= Sophora Microphylla, C.a= Carmichaelia australis.*

4.5 Conclusions

Work reported in this chapter investigated the influence of N-fixing bacteria on the growth of native and exotic legumes, and interactions with the growth of *P. amoena* (a non N-fixing plant). N-fixing bacteria were inoculated to the legumes, and the inoculants positively affected biomass of *C. proliferus* and nodulation of *C. proliferus* and *Astragalus cicer*. There was no siginificant effect of inoculation on biomass of the native legumes, but inoculation of *S. prostrata* enhanced soil available nitrogen. The non N-fixing species (*P. amoena*) was planted with different native and exotic N-fixers in pots, and the growth of *P. amoena* was greater with the native legumes. This may help to provide a recommendation for planting management of other native species, which may also benefit from association with native N-fixers.

Chapter 5

Response of native N-fixing species to different soil nutrient conditions

5.1 Introduction

New Zealand's 80 million years of geographic isolation has led to the evolution of a highly endemic native flora (Mittermeier et al., 1999). Conversion of land to agricultural use and the introduction of exotic species has subsequently resulted in the decline of native vegetation (Pawson et al., 2010; Walker et al., 2006). Current restoration practices in agricultural landscapes are taking place on soils that have been profoundly modified from their original physicochemical status. The rationale of the work reported in the present chapter was to explore the significance of this, particularly in the context of native nitrogen-fixing plants.

Most nutrients cycle from plant to soil to plant, which provides the foundation of ecosystem functionality. Nitrogen and phosphorus are two of the most important nutrients for plant growth (Vance, 2001). Nutrients that are strongly bound to the soil, such as PO_4^{3-} are relatively unaffected by leaching from rainwater, whilst weakly bound nutrients such as NO_3^- are more mobile and easily lost through leaching (Kabata-Pendias, 2010). In New Zealand imbalances of these elements through fertilizer usage and the livestock effluents has raised widespread environmental concerns in terms of soil health of soil and pollution of water (Scherr & McNeely, 2008).

Soil nitrogen availability is a major limitation to plant production, particularly in agricultural production systems, but may be less significant for natural plant communities (Lambers et al., 2008). Soil contains nitrogen in organic and inorganic forms, but only ammonium and nitrate are readily available to plants uptake (Lipson & Näsholm, 2001; Robertson & Groffman, 2007). These two forms of soil nitrogen are continually being removed by crops, and transformed and mobilized through leaching, erosion, nitrification and denitrification. Replenishment of soil nitrogen by microbial fixation alone is generally insufficient for non N-fixing plant growth (Eck & Jones, 1992; Jensen & Hauggaard-Nielsen, 2003b). Agricultural systems represent one end of a wide spectrum of N usage (Clark et al., 2007; Loganathan et al., 2003). A previous PhD student in the same research group at Lincon (Franklin, 2014), has found that some native plant species are tolerant of soil nitrogen but have no significant response to raised soil nitrogen or phosphorus, but little is known of the response of native nitrogen-fixing species to soil fertility.

The work reported in this chapter was an attempt to understand the relevance of the interaction of soil nutrients and native nitrogen-fixing plants in the context of land restoration in agriculture landscapes. The aims were to investigate the response of five New Zealand native species (four N-fixing and one non nitrogen-fixing native species) to variable soil nutrients in a glasshouse experiment.

5.2 Materials and Methods

5.2.1 Plants and soil preparation

The five native species used in this study included four native N-fixing species (Leguminosae: *S. microphylla*, *S. prostrata*, *C. australis* and Rhamnaceae: *D. toumatou*) and a non N-fixing native plant (Rhamnaceae: *P. amoena*). *Pomaderris amoena* is a rare and threatened species present in Eyrewell forest (the study site in this research) and little is known of the ecology of this species. All the plants were approximated one-year old and were purchased from Motukarara Department of Conservation Nursery, Canterbury. Soil was collected from the Eyrewell study site (0-15cm), at the edge of a pine forest with low concentrations of nitrogen (ammonium 0.02 mg kg^{-1}, nitrate 0.06 mg kg^{-1}) (-43.4236, 172.3116). The collected soil sieved through a 2mm sieve prior before the experiment setting up. Further details of Eyrewell soil properties are detailed in Appendix D.

5.2.2 Experiment design and plant maintance

Seven treatments were applied to all the native species in this experiment: control (C), low nitrogen (N100), high nitrogen (N300), phosphorus (P), lime (L), low nitrogen with lime (N100+L), and low nitrogen with lime and phosphorus (N100+L+P) (as detailed in Table 5.1). The seven treatments of the legumes (*S. microphylla*, *S. prostrata* and *C. australis*) all contain inoculated and non-inoculated group. Soil was used for all the treatments and inoculation did not sterilise, and contained naturally occurring microorganisms, including N-fixing bacteria. This means that any effect of inoculation described in this experiment is seen between soil with no additional inoculants and soil with inoculants. Every treatment included treatment with and without inoculation for the native species had 5 replicates. The legumes (*S. microphylla*, *S. prostrata* and *C. australis*) were inoculated with rhizobia strains of ICMP 19041, ICMP 19545 and ICMP 19535 which were known to be effective in these species (Tan, 2014). Further details of rhizobia and preparation are provided in Chapter 4 (#4.2.1.2). A total of 40ml $plant^{-1}$ rhizobia were applied for the legumes once planted.

Table 5.1 Treatments application for native species.

	Treatments							
Speices	C	N100	N300	P	L	N100+L	N100+L+P	Inoculation
N-fixing species								
Legminosae:								
Sophora microphylla	√	√	√	√	√	√	√	√
Sophora prostrata	√	√	√	√	√	√	√	√
Carmichaelia australis	√	√	√	√	√	√	√	√
Rhamnaceae:								
Discaria toumatou	√	√	√	√	√	√	√	—
Non N-fixing species								
Rhamnaceae:								
Pomaderris amoena	√	√	√	√	√	√	√	—

C=Control= no extra nutrients has been added, N100=100kg N ha^{-1}, N300=300kg N ha^{-1}, L=Lime= 6t lime ha^{-1}, P=Phosphorus=470kg superphosphate ha^{-1}, N100+L= 100kg N+ 6t lime ha^{-1}, N100+L+P= 100kg N+ 6t lime+ 470kg super phosphate ha^{-1}.

In all cases, fertilizer amendments were representative of those used in the conversion of the plantation forest to intensive irrigated farming systems (Eyrewell area). The low nitrogen (N100) treatment was equivalent to 100kg ha^{-1} of nitrogen on soil and high nitrogen (N300) treatment was equivalent to 300 kg ha^{-1}, applied as urea (46% N). Lime (L) and phosphorus (P) treatments were equivalent to 6 t ha^{-1} of horticulture lime ($CaCO_3$) and 470 kg ha^{-1} of superphosphate containing 9% P were applied. Horticulture lime and superphosphate were mixed with the collected soil and left for one week before planting. Solid urea was dissolved in water and the solution was applied four times as split applications during the first month once plants were planted. This experiment was maintained in a glasshouse at the Lincoln University Greenhouse Nursery (shown as Figure 5.1) with approximately 1025 hours sunlight (during the 6 months growth period) and an average temperature of 25°C during the daytime and 15°C at the night. The arrangement of pots was a fully randomised single-block experimental design.

Figure 5.1 The glasshouse experiment of native species (*S. microphylla, S. prostrata, C.australis, D. toumatou* and *P. amoena*) (Photograph, Shanshan Li).

5.2.3 Soil and plant measurement

Plant height, dry weight, and diameter of the main stem were measured after six months' growth. Soil measurements of NH_4^+ and NO_3^- concentrations, Olsen P, and soil pH were measured after six months' growth. Plant dry weight was determined after over drying at 60℃ for 72 hours. The increase in shoot height and main stem diameter were also measured. For measurement of soil NH_4^+ and NO_3^- concentration and soil pH refer to section 4.2.1.4. For Olsen P measurement (Olsen, 1954), 1 g of dry soil was suspended into 20 ml 0.5 mol. of $NaCO_3$ in 50 ml flask. The mixture was shaken for 30 minutes then centrifuged at 2,000 rpm for 10 minutes and filtered using Whatman No. 42 filter paper. The extracted solution was added to 10 ml filtrate and two drops of p-nitrophenol. The solution was mixed with sufficient 2 M H_2SO_4 to become clear. Deionized water was mixed with 5 ml Working Colour Reagent to make up 50 ml, then shaken well and left for half an hour. The extracted solutions were analysed by a UV/VIS (UV160A) spectrophotometer (Shimadu, Japan) at 880nm. Nitrogenase activity was measured for the legumes' nodules with and without inoculation. Ten nodules were randomly collected from each of the legumes, then measured following Acetylene Reduction Assay (ARA) method (Fishbeck et al., 1973) using gas chromatography (GC) at the National Centre for Nitrous Oxide Measurement (Lincoln University, New Zealand). Roots without nodules were measured as blank.

5.2.4 Statistical analysis

ANOVA (one-way) Fisher's comparisons (Minitab, 17 version) was used to determine any significant changes in plant biomass, soil ammonium, soil nitrate, soil pH and soil phosphorus between treatments, for each species. Data was first checked for normal distributions. Significant differences between species for each treatment was determined. Comparisons of soil ammonium, soil nitrate, soil pH and soil Olsen P of all species in each treatment were tested using ANOVA (one-way) Fisher's comparisons (Minitab, version 17). The interaction effects of treatments and species (treatments*species) on soil ammonium, soil nitrate, soil pH and soil phosphorus were tested using General Linear Model (ANOVA (two-way)) of Minitab (version 17). The interaction effects of species and inoculation (species*inoculation) on soil nitrogen, pH and Olsen P were tested using General Linear Model (ANOVA two-way, Minitab 17). The relationship between nitrogen application and mineral nitrogen; and the relationship between pH and soil Olsen P with nitrogen and lime treatments were analysed using linear regression (SigmaPlot 13.0).

Principal component analysis (PCA) was used for grouping the effects of treatments and investigating the relationship between the treatments and soil properties. The relationship between species, plant biomass and soil chemical property was tested by PCA using Minitab (version 17).

5.3 Results

5.3.1 Growth of plants

Pomaderris amoena showed a high biomass with nitrogen application on high (300 kg ha^{-1}) and low (100 kg ha^{-1}) nitrogen treatments ($p<0.001$). *Pomaderris amoena* and *C. australis* showed significant increases in dry biomass, shoot height and main stem diameter under N100+L+P treatment.

There was no significant difference in a combined shoot and root dry weight of *S. microphylla* and *S. prostrata* under any treatments (Table 5.2). The shoot dry weight of *D. toumatou* was higher under N100+L+P than N100, N300, P and N100+L ($p<0.05$), but there was no significant difference in root dry biomass. *Pomaderris amoena* and *C. australis* had significant differences in dry biomass ($p<0.01$, Table 5.2). *Carmichaelia australis* showed higher dry biomass under the L and N100+L+P treatments ($p<0.05$) compared to the control. Shoot and root dry weight of *P. amoena* was higher in nitrogen treatments than for the control, P and L ($p<0.001$).

The shoot height ($p < 0.05$) and main stem diameter of *P. amoena* were significantly higher ($p<0.001$) with nitrogen addition than with other treatments. There was no significant increase in shoot height, or main stem diameter in *D. toumatou* or *S. prostrata* (Table 5.3). Shoot height increase was lower for *C. australis* and *S. microphylla* under N300 treatment compared to N100+L+P treatment ($p<0.05$). Main stem diameter increase in *C. australis* was higher in the N100, L and N100+L+P treatments compared to the control ($p<0.001$).

Table 5.2 Mean (±SE) of shoot and root dry weight of the five native species in seven treatments after six months' growth. C=Control= no extra nutrients has been added, N100=100kg N ha^{-1}, N300=300kg N ha^{-1}, L=Lime= 6t lime ha^{-1}, P=Phosphorus=470kg super phosphate ha^{-1}, N+L= 100kg N+ 6t lime ha^{-1}, N+L+P= 100kg N+ 6t lime+ 470kg super phosphate ha^{-1}. The results were tested using ANOVA (one-way) Fisher's comparisons. Means that do not share same letters are in different groups for each column, n=5. *, **, * indicate the effect being significant at p<0.05, p<0.01, p<0.001.**

Species					
Dry weight (shoot) (g)					
Treatments	*P. amoena*	*D. toumatou*	*S. microphylla*	*S. prostrata*	*C. australis*
Control	7.0 (1.49)^{b}	5.95 (1.01)^{ab}	4.60 (0.52)^{b}	2.13 (0.58)^{c}	5.99 (0.36)^{c}
N100	17.66 (1.88)^{a}	4.16 (0.82)^{b}	6.17 (0.61)^{ab}	4.66 (0.65)^{a}	7.62 (1.00)^{bc}
N300	13.71 (0.71)^{a}	3.07 (0.76)^{b}	4.78 (0.44)^{b}	3.90 (0.67)^{bc}	7.11 (0.83)^{bc}
L	6.39 (0.41)^{b}	5.67 (0.81)^{ab}	4.51 (0.53)^{b}	3.48 (0.66)^{abc}	9.78 (0.78)^{b}
P	6.92 (1.09)^{b}	4.88 (0.78)^{b}	4.24 (0.60)^{b}	2.90 (0.30)^{abc}	8.28 (0.84)^{bc}
N100+L	15.86 (2.87)^{a}	5.18 (0.96)^{b}	8.14 (0.98)^{a}	2.82 (0.38)^{bc}	9.05 (1.79)^{bc}
N100+L+P	14.07 (2.35)^{a}	8.35 (1.39)^{a}	6.46 (1.92)^{ab}	4.16 (0.61)^{ab}	13.18 (1.29)^{a}
	***	*			**
Dry weight (root) (g)					
Treatments	*P. amoena*	*D. toumatou*	*S. microphylla*	*S. prostrata*	*C. australis*
Control	4.80 (0.44)^{b}	4.39 (1.03)^{ab}	2.42 (0.30)^{a}	1.42 (0.64)^{b}	2.97 (0.28)^{c}
N100	12.33 (3.01)^{a}	5.59 (1.15)^{a}	3.30 (0.42)^{a}	4.89 (0.97)^{a}	5.73 (0.76)^{a}
N300	9.366 (1.18)^{a}	1.43 (0.34)^{c}	2.17 (0.31)^{a}	3.28 (0.45)^{ab}	4.16 (0.65)^{abc}
L	4.21 (0.67)^{b}	3.01 (0.67)^{bc}	2.26 (0.31)^{a}	3.15 (0.63)^{ab}	5.81 (0.56)^{a}
P	4.37 (0.43)^{b}	3.37 (0.25)^{abc}	2.75 (0.42)^{a}	3.00 (0.48)^{b}	3.61 (0.49)^{bc}
N100+L	11.73 (1.62)^{a}	3.47 (0.70)^{abc}	3.25 (0.57)^{a}	2.77 (0.53)^{b}	5.06 (0.78)^{ab}
N100+L+P	10.78 (2.74)^{a}	3.59 (0.57)^{abc}	2.72 (0.61)^{a}	2.58 (0.41)^{b}	5.39 (0.55)^{a}
	***				*

Table 5.3 Mean (±SE) of shoot height and diameter (mian stem) increase (cm) of the five native species in seven treatments after six months' growth. C=Control= no extra nutrients has been added, N100=100kg N ha^{-1}, N300=300kg N ha^{-1}, L=Lime= 6t lime ha^{-1}, P=Phosphorus=470kg super phosphate ha^{-1}, N+L= 100kg N+ 6t lime ha^{-1}, N+L+P= 100kg N+ 6t lime+ 470kg super phosphate ha^{-1}. The results were tested using ANOVA (one-way) Fisher's comparisons. Means that do not share same letters are in different groups for each column, n=5. *, **, * indicate the effect being significant at p<0.05, p<0.01, p<0.001.**

	Species				
Shoot height increase (cm)					
Treatments	*P. amoena*	*D. toumatou*	*S. microphylla*	*S. prostrata*	*C. australis*
Control	1.8 (0.46)^{c}	31.4 (5.25)^{abc}	24.7 (5.14)^{ab}	10.0 (2.0)^{ab}	32.9 (4.30)^{bc}
N100	8.8 (0.67)^{ab}	22.7 (6.15)^{bc}	10.4 (2.24)^{b}	7.5 (1.55)^{ab}	30.2 (4.04)^{c}
N300	12.3 (5.37)^{a}	13.3 (2.18)^{c}	7.25 (2.01)^{b}	4.2 (0.58)^{b}	25.1 (2.94)^{c}
L	2.6 (0.85)^{bc}	46.2 (10.69)^{a}	28.6 (5.05)^{a}	14.8 (3.44)^{a}	37.8 (4.99)^{abc}
P	1.0 (0.50)^{c}	36.3 (5.01)^{abc}	24.8 (5.32)^{ab}	12.8 (2.78)^{a}	32.0 (4.27)^{bc}
N100+L	7.3 (4.56)^{abc}	30.6 (6.49)^{abc}	19.2 (7.91)^{ab}	8.6 (1.86)^{ab}	43.8 (5.71)^{ab}
N100+L+P	9.5 (2.02)^{a}	38.8 (7.85)^{ab}	29.0 (10.0)^{a}	14.2 (3.90)^{a}	46.8 (4.58)^{a}
	*		*		*
Diameter (main stem) increase (cm)					
Treatments	*P. amoena*	*D. toumatou*	*S. microphylla*	*S. prostrata*	*C. australis*
Control	0.03 (0.05)^{e}	0.32 (0.05)^{ab}	0.18 (0.02)^{ab}	0.17 (0.03)^{a}	0.24 (0.03)^{b}
N100	0.15 (0.03)^{bc}	0.25 (0.04)^{b}	0.23 (0.04)^{a}	0.15 (0.03)^{a}	0.38 (0.02)^{a}
N300	0.28 (0.03)^{a}	0.23 (0.03)^{b}	0.27 (0.01)^{a}	0.18 (0.02)^{a}	0.24 (0.02)^{b}
L	0.12 (0.03)^{cd}	0.32 (0.06)^{ab}	0.17 (0.03)^{ab}	0.21 (0.06)^{a}	0.39 (0.05)^{a}
P	0.06 (0.02)^{de}	0.25 (0.02)^{b}	0.10 (0.04)^{b}	0.13 (0.01)^{a}	0.21 (0.02)^{b}
N100+L	0.21 (0.04)^{ab}	0.26 (0.04)	0.25 (0.03)^{a}	0.15 (0.03)^{a}	0.18 (0.02)^{b}
N100+L+P	0.25 (0.03)^{a}	0.40 (0.04)	0.22 (0.05)^{a}	0.13 (0.02)^{a}	0.33 (0.03)^{a}
	***				***

5.3.2 Soil mineral nitrogen, pH and Olsen P

Soil properties were modified by different treatments and by different species. The concentrations of soil mineral nitrogen (NH_4^+ and NO_3^-), soil pH and soil Olsen P were all significantly affected by the treatments (p<0.001, Table 5.4) and the plant species used in the treatments (p<0.01, Table 5.4) (p<0.001, Table 5.4). In addition, soil properties were significant differences in different treatments for each species. The interaction effect of species and treatments showed significant differences in soil chemical properties (p<0.01, Table 5.4).

Table 5.4 P values of the effects of the five plant species and the seven treatments on soil chemical properties after six months' plants growth. The results were analysed using ANOVA (one-way/two-way) comparisons by Minitab 17.

	P values			
	Soil ammonium	Soil nitrate	Soil pH	Soil Olsen P
Species	<0.001	<0.001	<0.001	<0.001
Treatments	<0.01	<0.01	<0.001	<0.001
Species*Treatments	<0.01	<0.001	<0.001	<0.01

5.3.2.1 Soil nitrogen

Effects on plants

Both of the soil ammonium and nitrate levels with native plants were highest with the 300 kg ha^{-1} N treatment ($p<0.001$). N-fixers and non N-fixing plants provided different soil NO_3^- contents under low N (100 kg ha^{-1}) treatment.

Soil NH_4^+ under N300 treatment was the highest for all species after six months' growth ($p<0.001$, Figure 5.2a). There was no significant difference of the soil NH_4^+ in the other treatments (C, N100, L, P, N+L, N+L+P) between the native plant species.

Soil NH_4^+ and NO_3^- contents using the non N-fixing species *P. amoena*, were highest under the N300 treatment ($p<0.001$). For the N-fixing species, the soil NO_3^- showed more variability between the treatments than did the soil NH_4^+. *Sophora microphylla* and *S. prostrata* had same patterns for the soil NO_3^- levels under the different treatments. The N300 treatment led to the highest soil nitrate level ($p<0.001$, Figure 5.2b); N100, N100+L and N100+L+P treatments led to lower soil nitrate than N300 but higher than C, L and P treatments ($p<0.001$) for the two *Sophora* spp. For *D. toumatou* and *C. australis*, N300 treatment also provided the highest soil NO_3^- contents but there was no significant difference between the C, L, P and N+L+P treatments which differed to the two *Sophora* species (Figure 5.2b).

Effect of plants on soil mineral nitrogen

Plant species affected soil nitrogen (NH_4^+ and NO_3^-) in C, and N100 treatments ($p<0.05$). The highest soil NH_4^+ content under the C, L and P treatments was using *S. microphylla* ($p<0.001$, Figure 5.3). Under low N applications, including single N and its combination treatments with lime and P, the soil NH_4^+ of *S. microphylla* was higher than *P. amoena* and *D. toumatou* ($p<0.001$), but there was no difference between the two *Sophora* spp.. In addition, there were no significant differences under the N300 treatment between all the N-fixing species and *P. amoena*.

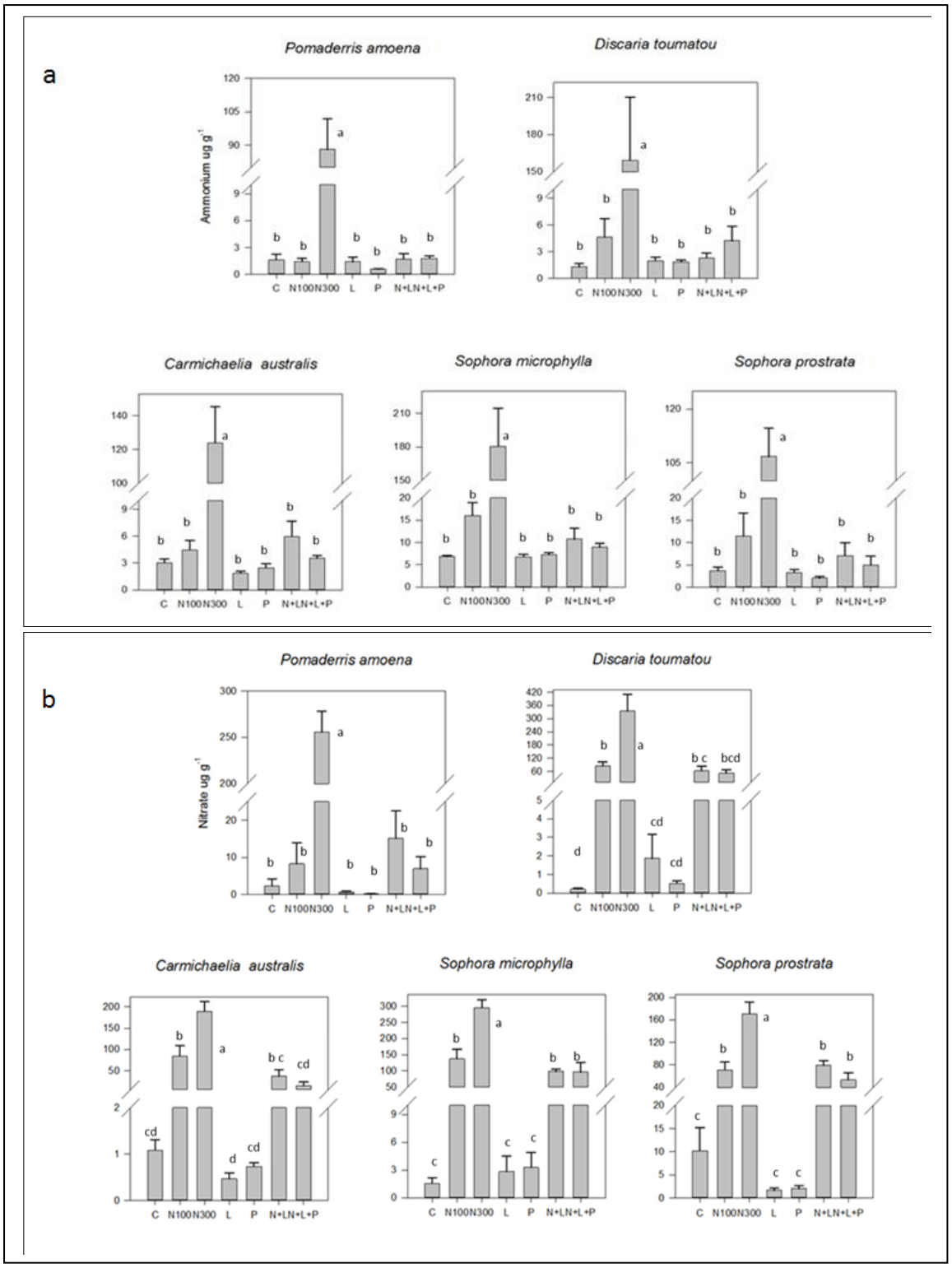

Figure 5.2 Mean (±SE) soil ammonium and nitrate contents under the seven different treatments by each species. C=Control= no extra nutrients has been added, N100=100kg N ha^{-1}, N300=300kg N ha^{-1}, L=Lime= 6t lime ha^{-1}, P=Phosphorus=470kg super phosphate ha^{-1}, N+L= 100kg N+ 6t lime ha^{-1}, N+L+P= 100kg N+ 6t lime+ 470kg super phosphate ha^{-1}. The results were tested using ANOVA (one-way) Fisher's comparisons, n=5, p<0.001. Means that do not share same letters are in different groups for each species. The bars which share letters are not significantly different.

Sophora microphylla generated higher soil NH_4^+ in the most of the treatments after six months' growth. Soil NO_3^- under control treatment was significantly higher with *S. prostrata* ($p<0.05$). Under N100 treatment, soil NO_3^- was lower in *P. amoena* than with *D. toumatou, S. microphylla* and *C. australis* ($p<0.05$, Figure 5.3). For the N-fixing species, *D. toumatou* and *S. microphylla* generated higher soil NO_3^- than *S. prostrata* and *C. australis* with the N300 treatment.

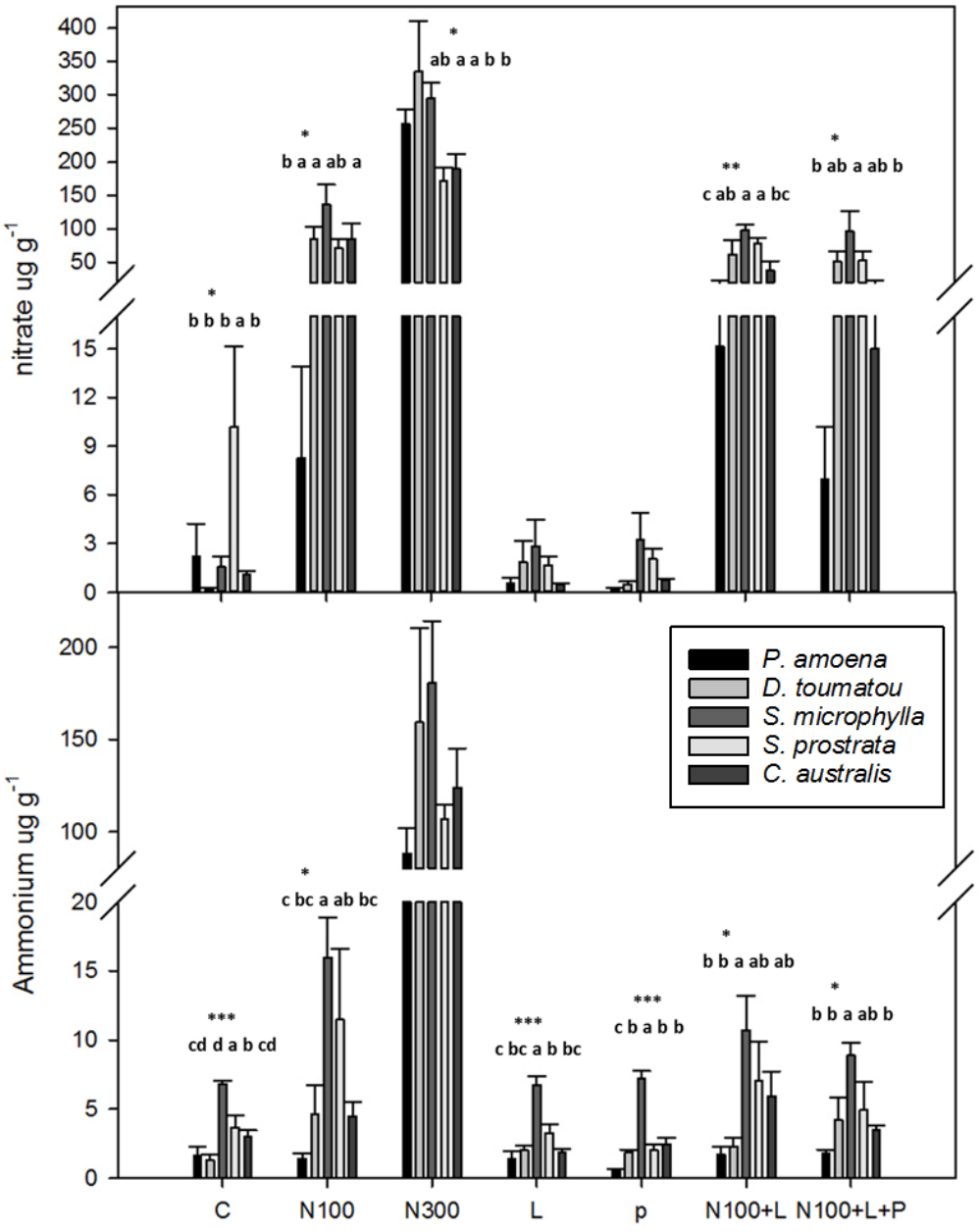

Figure 5.3 Mean (± SE) of soil mineral nitrogen (ammonium and nitrate) of the five species under the seven different treatments after six months' growth. C=Control= no extra nutrient has been added, N100=100kg N ha^{-1}, N300=300kg N ha^{-1}, L=Lime= 6t lime ha^{-1}, P=Phosphorus=470kg super phosphate ha^{-1}, N+L= 100kg N+ 6t lime ha^{-1}, N+L+P= 100kg N+6t lime+470kg super phosphate ha^{-1}. The results were tested using ANOVA (one-way) Fisher's comparisons. Means that do not share same letters are in different groups for each treatment, n=5. *, **, * indicate the effect being significant at p<0.05, p<0.01, p<0.001.**

5.3.2.2 Soil pH

Soil became more acid with 100kg ha^{-1} nitrogen (except *P. amoena*) and 300kg ha^{-1} nitrogen treatment for all the native species after six months' growth.

Soil pH was the lowest with *P. amoena* in the N300 treatment (P<0.001, Table 5.5). Soils with *P. amoena* under L, C and N100 treatments had higher pH than with N300, P and N00+L treatments (P<0.001). For the other four N-fixing species, soil under L treatments produced higher soil pH than the treatments with nitrogen applications (P<0.001, Table 5.5). Soil pH increased with lime application but not significant compared to the control group (Table 5.5).

There were significant differences in soil pH between the plant species with the 100 kg ha^{-1} N treatment (p<0.05, Figure 5.4). Under the 100 kg ha^{-1} N treatment, *P. amoena* gave higher soil pH values than *D. toumatou* and the two *Sophora* species (p<0.05, Figure 5.4). *Pomaderris amoena* also gave higher soil pH values than *D. toumatou* and the two *Sophora* species, under the combination treatments (100kg N+ 6t lime ha^{-1} and 100kg N+ 6t lime+ 470kg super phosphate ha^{-1}, p<0.001). Therefore, the non N-fixing plant *P. amoena* led to a higher soil pH than the N-fixing species (except *C. australis*) under low N treatments (p<0.05, Figure 5.4).

Table 5.5 Mean (± SE) of soil pH values for the five species under seven treatments after six months' plant growth. Control= no extra nutrient has been added, N100=100kg N ha^{-1}, N300=300kg N ha^{-1}, L=Lime= 6t lime ha^{-1}, P=Phosphorus=470kg super phosphate ha^{-1}, N+L= 100kg N+ 6t lime ha^{-1}, N+L+P= 100kg N+ 6t lime+ 470kg super phosphate ha^{-1}. The results were tested using ANOVA (one-way) Fisher's comparisons. Means that do not share same letters are in different groups for each column, n=5. *, **, * indicate the effect being significant at p<0.05, p<0.01, p<0.001.**

	Species				
Soil pH values					
Treatments	*P. amoena*	*D. toumatou*	*S. microphylla*	*S. prostrata*	*C. australis*
Control	5.08 ± 0.05 ^{ab}	5.02± 0.06 ^{a}	4.97± 0.13^{ab}	4.90± 0.21^{a}	5.10± 0.04^{ab}
N100	5.08± 0.08 ^{ab}	4.36± 0.07 ^{d}	4.49± 0.18^{c}	4.41± 0.05^{c}	4.72± 0.16^{c}
N300	4.05± 0.03 ^{c}	4.08± 0.07 ^{e}	4.14±0.04^{d}	4.03± 0.05^{d}	4.20± 0.06^{d}
L	5.23± 0.06 ^{a}	5.16± 0.02 ^{a}	5.19±0.05^{a}	5.33± 0.04^{a}	5.27± 0.04^{a}
P	4.97± 0.02 ^{b}	4.83± 0.03 ^{b}	4.90± 0.04^{b}	4.88± 0.01^{a}	4.70± 0.01^{c}
N100+L	5.03± 0.09 ^{b}	4.66± 0.04 ^{c}	4.44± 0.07^{cd}	4.52± 0.04^{bc}	4.86± 0.12^{bc}
N100+L+P	5.13± 0.09 ^{ab}	4.64± 0.07 ^{c}	4.56±0.07^{c}	4.66± 0.06^{b}	4.95± 0.06^{bc}
	***	***	***	***	***

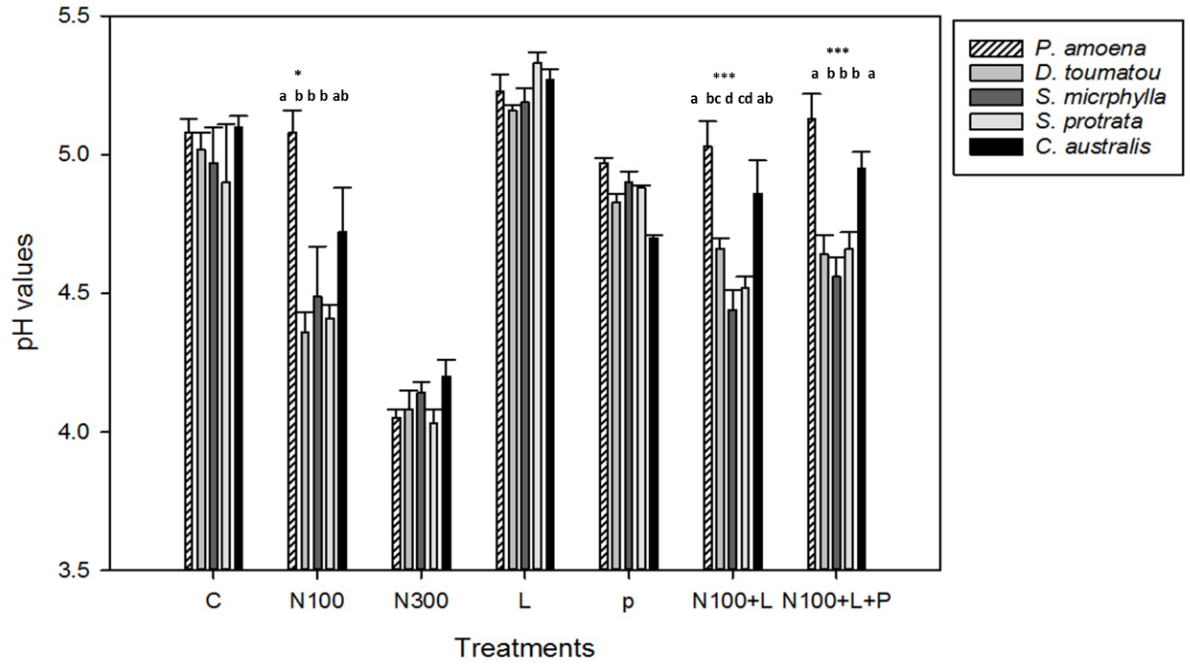

Figure 5.4 Mean (± SE) of soil pH values for the five plant species under each treatment after six months' growth. C=Control= no extra nutrient has been added, N100=100kg N ha^{-1}, N300=300kg N ha^{-1}, L=Lime= 6t lime ha^{-1}, P=Phosphorus=470kg super phosphate ha^{-1}, N+L= 100kg N+ 6t lime ha^{-1}, N+L+P= 100kg N+ 6t lime+ 470kg super phosphate ha^{-1}. The results were tested using ANOVA (one-way) Fisher's comparisons. Means that do not share same letters are in different groups for each treatment, n=5. *, **, * indicate the effect being significant at p<0.05, p<0.01, p<0.001.**

5.3.2.3 Soil Olsen P

High nitrogen treatment (300 kg ha^{-1} N) led to higher soil Olsen P levels than the control and L treatments for the native N-fixing species after 6 months' growth (Table 5.6). Pomaderris amoena generated the lowest soil Olsen P contents with 100 kg ha^{-1} N treatments (Table 5.6). Soil Olsen P values were much higher in the P and N100+L+P treatments than the other treatments, for all the native species (P<0.001, Table 5.6).

Although soil Olsen P contents varied under the different treatments (except single P treatment), *S. prostrata* provided higher soil Olsen P levels in most of the soil conditions. Under the control treatment, *P. amoena* and *S. prostrata* had higher soil Olsen P than *D. toumatou* and *C. australis* (p<0.01, Figure 5.5). Soil Olsen P of *P. amoena* and *C. australis* was lower than the other N-fixing species under the N100 treatment (p<0.001). Under N300 (p<0.05) and N100+L+P (p<0.001) treatments, *S. prostrata* gave the highest soil Olsen P from all the species. *Sophora microphylla* generated a higher soil Olsen P than *D. toumatou* and *C. australis* in lime treatment (p<0.05, Figure 5.5). There was no significant difference between plant species with the single P treatment. *Sophora*

prostrata generated higher soil Olsen P than *P. amoena*, *D. toumatou* and *C. australis* under N100+L+P ($p<0.05$) but no significant difference compared to *S. microphylla*.

Table 5.6 Mean (± SE) of soil Olsen P values in different treatments by each plant species after six months' growth. Control= no extra nutrient has been added, N100=100kg N ha^{-1}, N300=300kg N ha^{-1}, L=Lime= 6t lime ha^{-1}, P=Phosphorus=470kg super phosphate ha^{-1}, N+L= 100kg N+ 6t lime ha^{-1}, N+L+P= 100kg N+ 6t lime+ 470kg super phosphate ha^{-1}. The results were tested using ANOVA (one-way) Fisher's comparisons. Means that not share same letters are in different groups for each column, n=5. *, **, * indicate the effect being significant at p<0.05, p<0.01, p<0.001.**

	Species				
Soil Olsen P (ug g-^{1})					
Treatments	*P. amoena*	*D. toumatou*	*S. microphylla*	*S. prostrata*	*C. australis*
Control	6.36 (0.31)^{cd}	4.51 (0.17)^{c}	5.61 (0.48)^{c}	6.29 (0.66)^{bc}	4.64 (0.10)^{de}
N100	4.44 (0.20)^{e}	5.69 (0.18)^{bc}	6.41 (0.17)^{bc}	6.34 (0.07)^{bc}	5.60 (0.23)^{d}
N300	6.84 (0.31)^{c}	6.93 (0)^{b}	7.45 (0.38)^{b}	8.16 (0.23)^{b}	7.27 (0.20)^{c}
L	5.48 (0.33)^{de}	4.56 (0.11)^{c}	5.54 (0.39)^{c}	5.21 (0.47)^{c}	4.14 (0.23)^{e}
P	15.42 (0.58)^{a}	14.10 (0.58)^{a}	14.38(0.49)^{a}	14.94 (0.68)^{a}	14.17(1.02)^{a}
N100+L	4.39 (0.24)^{e}	5.38 (0.30)^{bc}	5.82 (0.13)^{c}	6.58 (0.31)^{bc}	4.99 (0.18)^{de}
N100+L+P	12.66 (0.56)^{b}	13.52 (0.97)^{a}	14.76(0.76)^{a}	16.11(0.96)^{a}	11.83(0.53)^{b}
	***	***	***	***	***

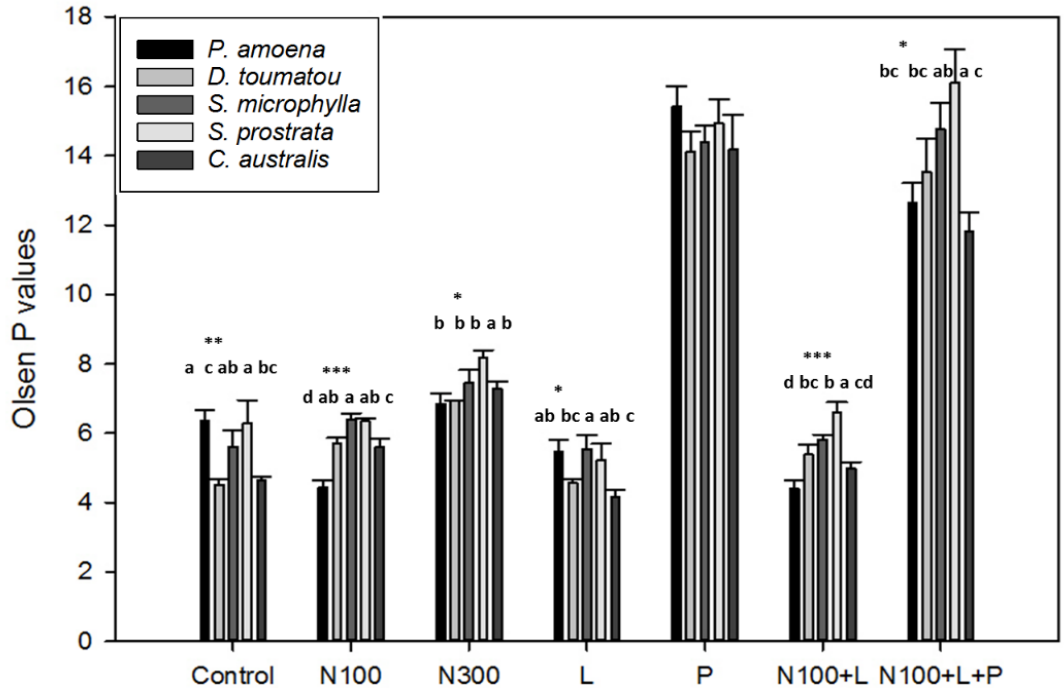

Figure 5.5 Mean (±SE) Olsen P values of the five plant species under each treatment after six months' growth. Control= no extra nutrient has been added, N100=100kg N ha^{-1}, N300=300kg N ha^{-1}, Lime= 6t lime ha^{-1}, Phosphorus=470kg super phosphate ha^{-1}, N100+L= 100kg N+ 6t lime ha^{-1}, N100+L+P= 100kg N+ 6t lime+ 470kg super phosphate ha^{-1}. The results were tested using ANOVA (one-way) Fisher's comparisons. For each treatment the bars which share letters are not significantly different. *, **, * indicates the effect being significant at p<0.05, p<0.01, p<0.001.**

5.3.3 Inter-relationship between soil properties and plants

5.3.3.1 Soil pH and nitrogen application

Soil pH decreased with increasing nitrogen application rates (Figure 5.6). Soil pH values ranged from 4.7 to 5.2 with no added N between all the plant species. Under the 100 kg ha^{-1} nitrogen treatment, soil pH varied dependant on the plant species. Soil pH ranged from 4.0 to 4.2 with 300 kg ha^{-1} N application between all the species (Figure 5.6).

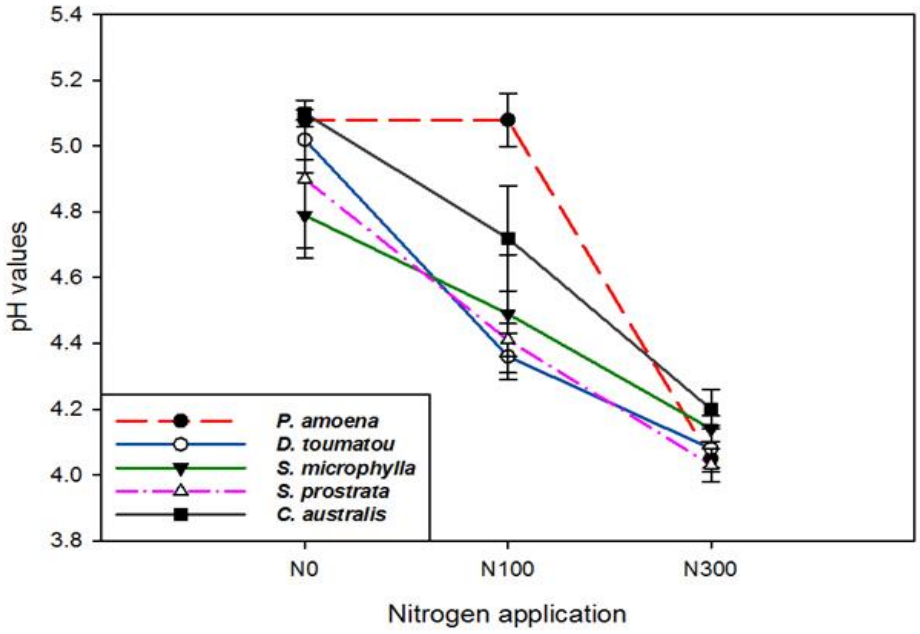

Figure 5.6 Mean (± SE) of soil pH values of five native species with different nitrogen application rates (n=5). N0=Control= no nitrogen added, N100=100kg N ha^{-1}, N300=300kg N ha^{-1}.

With increasing soil pH, soil mineral N included NH_4^+ and NO_3^- both decreased. Soil NO_3^- was higher than soil NH_4^+ when the soil pH ranged from 3.8 to 4.8 (Figure 5.7). The soil NH_4^+ and soil NO_3^- reached similar levels when soil pH values were over 4.8 (Figure 5.7). Soil pH decreased with increasing rates of soil N application (Figure 5.6), and soil mineral N levels increased with the addition of nitrogen (described in #5.3.2.1). Therefore, soil pH and soil mineral nitrogen are inversely correlated.

5.3.3.2 Soil pH and Olsen P for N-fixers

With the growth of the native N-fixing plants in N treatments, soil Olsen P decreased with increasing soil pH values (Figure 5.8). N300 application gave higher soil Olsen P values than the N100 and N100+L treatments. Nitrogen applications led to a decreasing soil pH for the N-fixing species (described in #5.3.3.1). Therefore, soil Olsen P responds positively to nitrogen applications and negatively to increased soil pH in the presence of the native N-fixing plants.

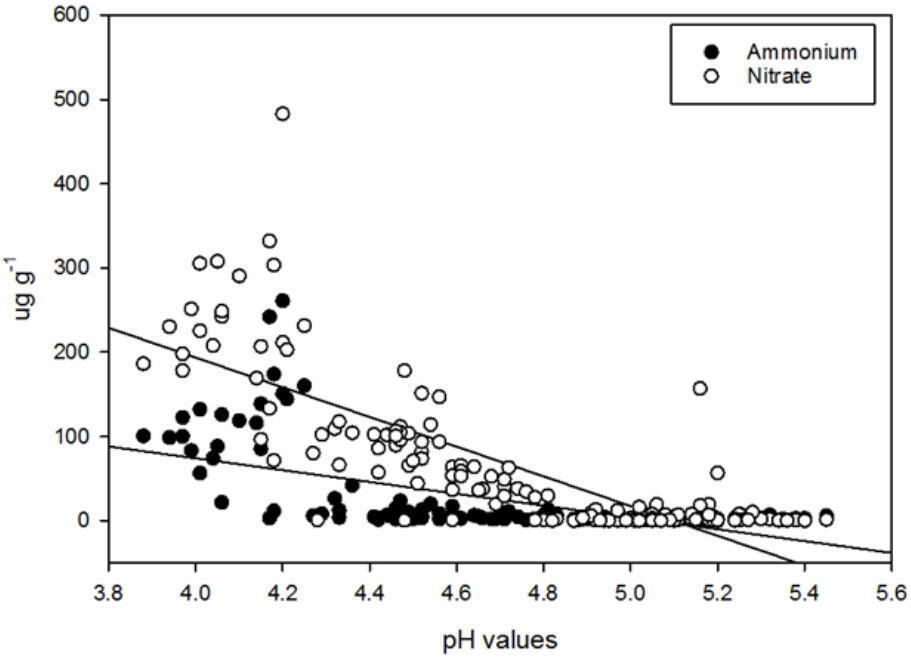

Figure 5.7 The relationship between soil pH values and soil mineral nitrogen (ammonium and nitrate) under the seven treatments. Data analysed using linear regression model. The soil ammonium Adjusted R^2=0.418, p<0.001; the soil nitrate Adjusted R^2=0.632, p<0.001.

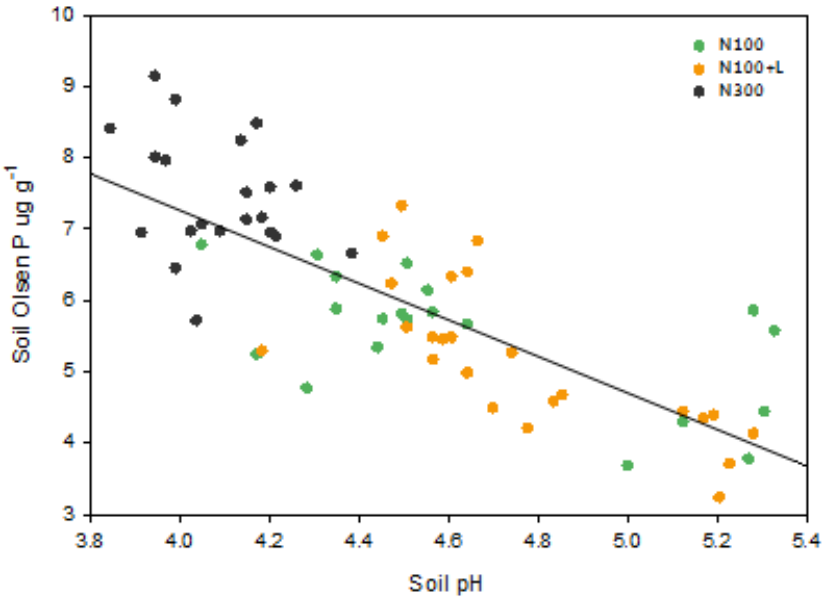

Figure 5.8 Relationship between soil pH values and soil Olsen P of the four N-fixing species with N applications. N100=100kg N ha^{-1}, N300=300kg N ha^{-1}, N100+L=100kg N+6t CaCO3 ha^{-1}. Data analysed using linear regression model, Adjusted R^2=0.6263, p<0.0001.

5.3.4 Principal Components Analysis

5.3.4.1 Treatments grouping and soil chemical property

The treatments were partitioned into four groups by PCA analysis for soil ammonium, nitrate, pH and Olsen P (Figure 5.9). In the first component, treatment N300 was positively related to the increasing soil mineral nitrogen ($NH4^+$ and $NO3^-$). Control and Lime treatments were negatively related to the decreasing soil pH. Treatments of N100 and N100+L were related to the soil pH and soil mineral nitrogen ($NH4^+$ and $NO3^-$). In the second component analysis, P and N100+L+P were positively related to increased soil Olsen P.

In terms of the above results, the 7 treatments were partitioned into four groups: Group 1: P and N100+L+P treatments, Group 2: Control and Lime treatments, Group 3: N100 and N100+L treatments, Group 4: N300 treatment, for the following analysis.

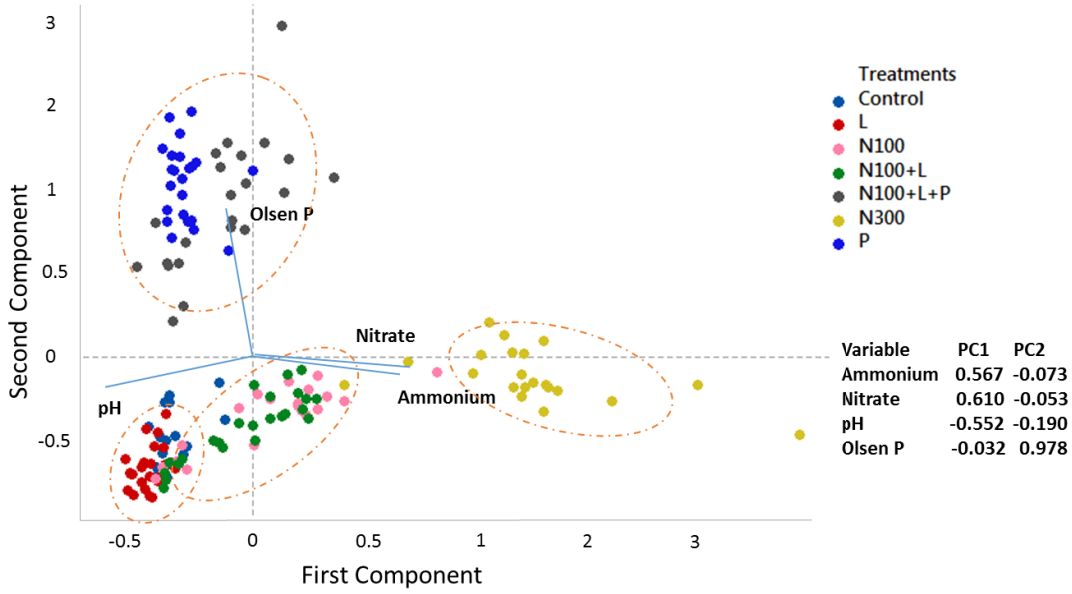

Figure 5.9 Principal component analysis of soil chemical property (soil $NH4^+$, $NO3^-$, pH and Olsen P). The seven treatments shown in different colours. Eigenvalues of the first and second component were 2.51 and 1.03 respectively.

5.3.4.2 Plant species, dry biomass and soil chemical properties

A Principal Component Analysis of plant dry biomass and soil chemical properties relating to the five native plant species was carried out (Figure 5.10). In different soil nutrient conditions, *P. amoena* was associated with increased plant dry biomass and *S. microphylla* with soil ammonium. The other three species responded differently to differing soil conditions.

Carmichaelia australis was positively associated with plant dry biomass (shoot and root) and soil pH in Treatment groups 1 and 2. *Discaria toumatou* was related to soil nitrogen and Olsen P in group1 but in group 2 was more associated with soil pH. *Pomaderris amoena* was related to plant dry biomass in group 1, 3 and 4. In group 2 (Control and Lime treatments), *P. amoena* was more related to soil Olsen P and root dry biomass. *Sophora microphylla* responded to soil ammonium in all the groups, and responded to soil nitrate in group 1 and 4. *Sophora prostrata* was more associated with soil Olsen P in group 1 and 4, but was more strongly associated with soil pH and soil ammonium in group 2.

5.3.5 The effects of inoculation on native legume species (*S. microphylla, S. prostrata, C. australis*)

5.3.5.1 Inoculation and plant dry biomass

There was no significant difference on biomass for the two *Sophora* spp. with inoculation compared to no inoculants addition. However, under inoculation soil conditions, *S. prostrata and C. australis* had different responses to different soil nutrient treatments. The N100+L+P treatment provided higher shoot dry biomass than in the other treatments ($p<0.01$, data not shown).

In *S. microphylla*, there was no significant difference between with and without inoculation in terms of shoot or root dry biomass in any treatment. For *S. prostrata* and *C. australis*, there was no significant difference in the root dry weight between inoculation and non-inoculation treatments but a significant difference in the shoot dry weight (g) for *C. australis* under L and N100+L treatments, the shoot dry biomass was lower with inoculation than without ($p<0.01$, data not shown). With inoculation, the shoot dry weight of *S. prostrata* in N100+L+P treatment was higher than the control, N100 and P treatments ($P<0.05$), and the dry shoot biomass of *C. australis* under the N100+L+P treatment was higher than the control, L, and N100+L treatments ($p<0.01$) (data not shown).

5.3.5.2 Inoculation and soil nutrients

There was only one significant effect between inoculation and non-inoculation on soil properties from all the treatments: Soil pH decreased with inoculated legume species under L treatment ($p<0.05$). The interaction effects of species and inoculation were significant on soil Olsen P under P and N100+P+L treatments ($p<0.01$, data not shown).

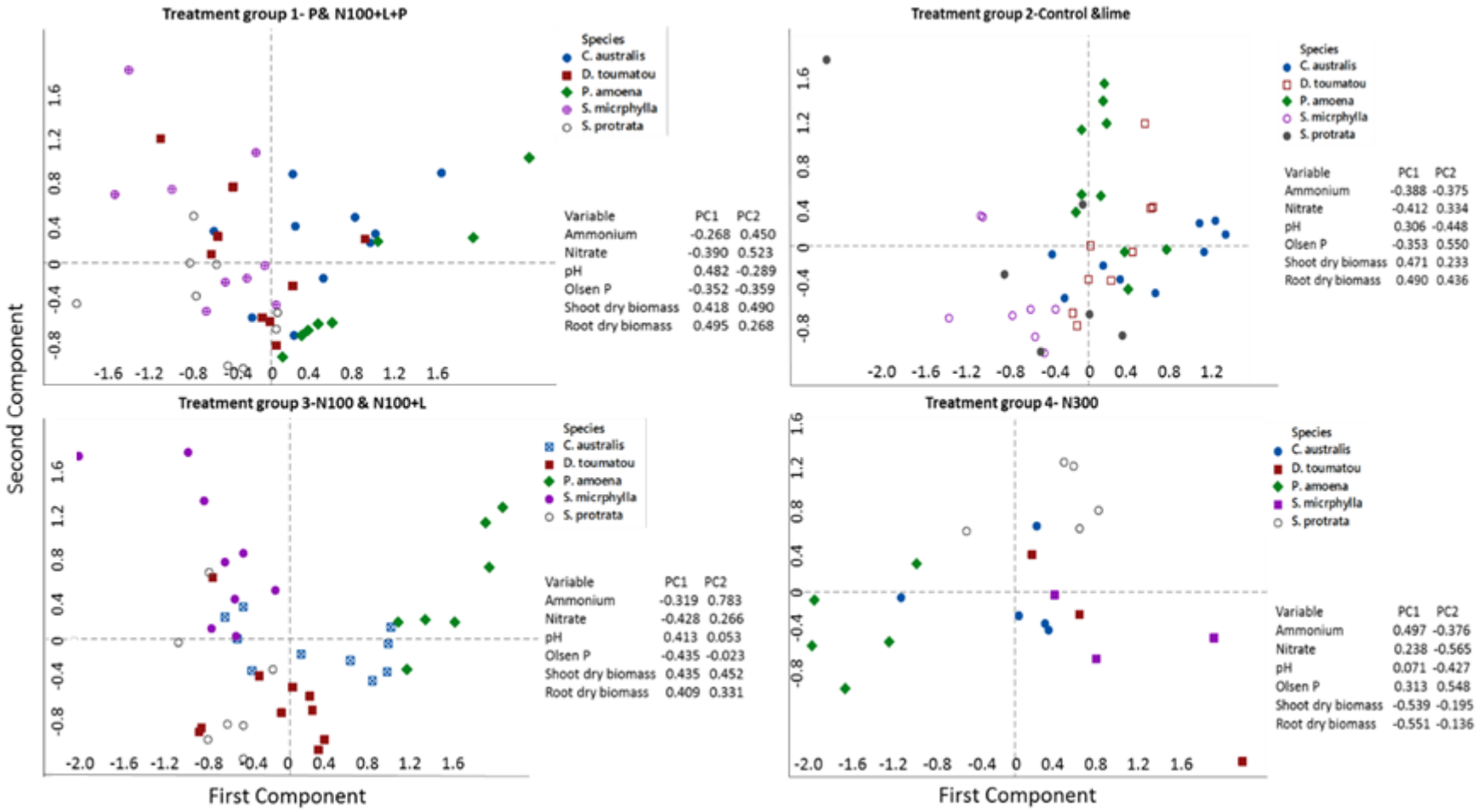

Figure 5.10 Principal component analysis of plant dry biomass and soil chemical properties. Species are shown in different shapes and colours.

No nodules were found with any inoculated or non-inoculated N treatments, including low N (100 kg ha^{-1}), high N (300 kg ha^{-1}) and their combined treatments with lime and P (Figure 5.11). Nodules were found in the control, lime, phosphorus treatments with and without inoculation. Nodules were bigger with phosphorus application than control and lime treatments (Figure 5.11). Additionally, there was no significant difference of nodule nitrogenase activity between the legumes with and without inoculation, but nitrogenase activity was higher ($p<0.05$) when plants received phosphorus compared to the control (data not shown).

Figure 5.11 Roots of *Sophora microphylla* from different treatments. C=Control= no extra nutrients were added, LowN =100kg N ha^{-1}, L: lime, P=470kg super phosphate ha^{-1}, (I)=inoculation, Sm=*Sophora microphylla.* The number which follows "Sm" means replicate number of the species.

5.4 Discussion

5.4.1 Plant response to soil nitrogen

A higher biomass was attained by *P. amoena* with nitrogen application than any of the other nitrogen-fixing plants. *Pomaderris amoena* had increased dry weight, shoot height and main stem in response to nitrogen treatment. Some research reports that nitrogen application leads to increased

plant biomass in non N-fixing species (Amanullah et al., 2009; Awale, 2010; Zhibin et al., 2008). For the N-fixing species, *Carmichaelia australis* had higher dry biomass using combined and lime treatments than with the control. This increase in *C. australis*, dry biomass was not seen in the other three native N-fixing species. Chaudhry et al. (1999) reported that the yield and biomass of summer legumes were not significantly affected by nitrogen application. In the present study, there were no significant differences on biomass between nitrogen (100 kg ha^{-1} and 300 kg ha^{-1} nitrogen) applications and the control group for all the legume species. This as an important finding that no negative effect of additional soil nitrogen (equivalent to the highest agricultural inputs of 300 kg ha^{-1}) on the native legumes.

In the present study, all the five plant species had higher soil ammonium and nitrate contents using the 300 kg ha^{-1} nitrogen application than with other treatments. However, with soil nitrogen treatment of under 100 kg ha^{-1}, the soil ammonium and nitrate levels responded differently dependant on the plant species. There was no change in soil ammonium levels at nitrogen treatment of under 100 kg ha^{-1} for all plant species, and no significant difference in soil nitrate contents for *P. amoena* between the 100 kg ha^{-1} nitrogen applications and the control. However, the other four N-fixing species had higher soil nitrate contents with 100 kg ha^{-1} nitrogen applications than the control, which differed to the non N-fixing species *P. amoena*. Therefore, *P. amoena* absorbed more nitrogen from the soil than the other four N-fixing species when under the 100 kg ha^{-1} nitrogen treatments. Other studies have found that legumes could reduce nitrogen fertilizer usage (Frink et al., 1999; Jensen & Hauggaard-Nielsen, 2003a; McVay et al., 1989). In this research, the four N-fixing species led to a higher soil nitrate level with 100 kg ha^{-1} nitrogen application than the non N-fixing species (*P. amoena*). This may be because N-fixing species fixed some nitrogen following the 100 kg ha^{-1} nitrogen application, which reduced the absorption rate of nitrogen directly from the soil. Therefore, although some nitrogen fertilizer was added to the soil in the early stages (urea converted to mineral nitrogen), the legumes were still able to fix nitrogen through the process of nitrogen fixation, and they may not need to absorb as much nitrogen as non N-fixers.

5.4.2 Soil pH and nitrogen application

Soil acidity was altered by nitrogen (urea) application. The reason may be that urea can be converted to ammonium by hydrolysis (Cabrera et al., 1991), and ammonium can be oxidised to nitrite by chemoautotrophic bacteria and then oxidised to nitrate by *Nitrobacter*, through nitrification (Foth & Ellis, 1997). Under natural conditions, nitrate is most readily available in soil, which is the main form that can be taken up by plants. However, nitrification normally can be

inhibited by acidity in low pH soil conditions, which causes ammonium to become the main form of nitrogen available to plants (Foth & Ellis, 1997). The chemical reaction is represented below:

$$NH4^{+}\text{ (ammonium)} + O_2 \xrightarrow[\text{Nitrobacteria}]{\text{Nitrosobacteria}} NO_3^{-}\text{ (nitrate)} + 2H^{+} + H_2O.$$

The process of ammonium conversion releases hydrogen ions into the soil which leads to soil acidity. The relationship between soil pH values and soil mineral nitrogen (results in #4.3.3) showed soil nitrate levels are higher than ammonium in soil pH 4.0 - 4.8 (nitrogen addition caused decreased soil pH). There were no differences in soil nitrate and soil ammonium levels with soil pH of 4.8 to 5.4. This could be due to the excess nitrogen in the soil that the plants couldn't use at all. Therefore, the bacteria converted some ammonium to nitrate that led to a soil acidity level higher than in the soil which did not have extra nitrogen applied.

Soil Olsen P decreased with increasing soil pH (4.0-5.4) in the present research, indicating that soil Olsen P levels became higher when soil acidity increased by adding N (urea). This may be due to nitrogen application reducing nitrogen fixation by N-fixers. Lower nitrogen fixation would lower the requirement for P as phosphorus is an important component in the creation of the high levels of Adenosine Triphosphate (ATP) needed for nitrogen fixation (Olivera et al., 2004). Therefore, plants absorb less phosphorus from soil when nitrogen is added, which leads to more available P in the soil. Soil acidity also affects plants and microorganisms. Some rhizobia tolerate different levels of soil pH, but relatively few grow well under pH 4.5-5.0 (Mohammadi et al., 2012). The negative effects of soil acidity on plants and microbes are due to a disruption of signal exchanges of symbionts and depression of nodulation genes (Mohammadi et al., 2012). However, in this present study, the biomass of the native N-fixers was not influenced by soil acidity associated with the nitrogen applied to the soil.

5.4.3 Nodulation and fertilizer

Nodules were not found when plants received nitrogen fertilizer (100 and 300 kg ha^{-1} nitrogen and treatments which contained nitrogen). Andrew (1976) reported no nodulation in tropical and temperate pasture legumes with nitrogen application. Some research showed that nitrates inhibit nitrogen fixation by N-fixing plants (Waterer & Vessey, 1993), and that mineral nitrogen inhibited nitrogen fixation of peas, but only In relation to the beginning of nodulation (Voisin et al., 2002). Inhibitory effects on nodulation and nitrogen fixation in soybeans were evident when nitrogen was applied in greater than 5mm concentrations, but less so at lower concentrations (Ruschel et al.,

1979). Therefore, nitrogen fertilizer applications reduce nodulation in N-fixing species. N-fixing plants may directly absorb inorganic nitrogen from the soil when it contains high levels of mineral nitrogen. Increased soil acidity brought about by nitrogen addition, and subsequent lower nitrogen fixation levels may affect the rhizobia levels in the soil (Foy, 1984; Ibekwe et al., 1997) by nitrogen (urea) addition (explained in 5.4.2). Nodules of N-fixing species were larger with phosphorus treatment, and nitrogenase activity of nodules was higher in phosphorus soil in the present study. Another study showed phosphorous and manure addition on soil can improve nodulation (Otieno et al., 2007). This improvement in nodulation maybe due to the high level of ATP requirement by nitrogen fixation (Olivera et al., 2004). Some studies report that nodules are nutrient sinks for phosphorus, that nitrogen fixation is greatly affected by phosphorus (Adu-Gyamfi et al., 1989; Hart, 1989; Saxena & Rewari, 1991). However, inoculation of legume species did not affect plant growth in the present study. This may because the soil already contained N-fixing bacteria, and the extra inoculants did not contribute much to plant growth. Additionally, soil pH was decreased with inoculation under lime treatment for the three legume species. This may be due to dead microbes from inoculation increased soil organic matter, then under lime treatment (with a higher soil pH), some other bacteria were more active led to decomposition increased which naturally added acid to the soil. Moreover, nodules were found from the N-fixing plants with soil pH about 5.0 in the control group, indicating that rhizobia those associated with the legumes may also tolerate acid soil.

5.4.4 Nitrogen released by native N-fixing plants

The N-fixing species in this research fixed different amounts of nitrogen from the atmosphere. *Sophora microphylla* contributed higher ammonium into the soil than the other species (Figure 5.12, $p<0.001$), whereas *Sophora prostrata* released the most nitrate into the soil ($p<0.05$). *Carmichaelia australis* contributed more soil ammonium than *Pomaderris amoena*, but not significantly more than *D. toumatou* or *P. amoena*. Compared to *P. amoena*, *S. microphylla* and *S.a prostrata* contributed more soil mineral nitrogen at 14.04 and 9.82 kg ha^{-1} $annum^{-1}$ respectively. Although *P.amoena* did not contribute any soil mineral N, as would be expected, the increase in its soil N content may be due to the decomposition of organic matter (Van-Veen & Kuikman, 1990) and the effects of soil nitrifying bacteria releasing some mineral N into the soil. Some studies have reported that N-fixing crops and oats contribute more than 20% of inorganic N into soil (Wichern et al., 2007).

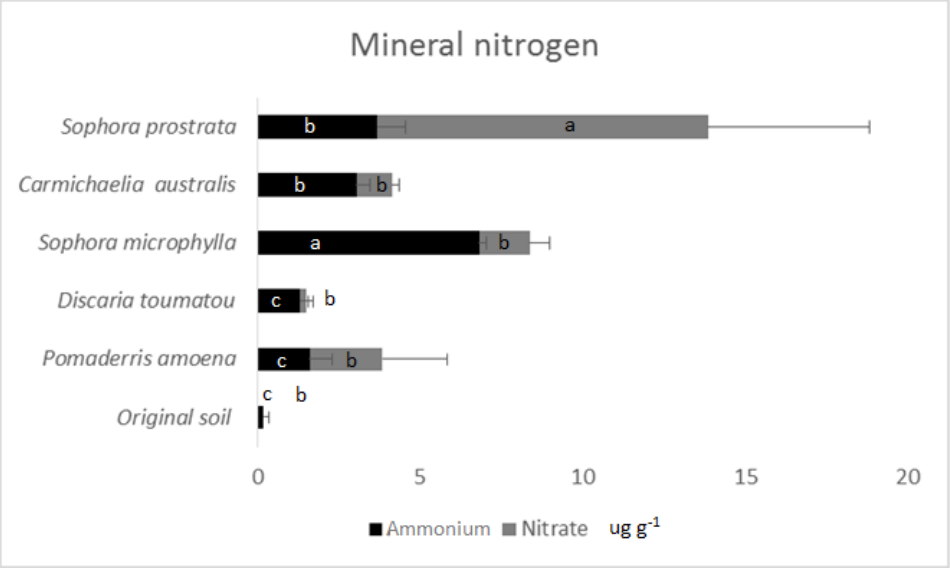

Figure 5.12 Nitrogen input from the native N-fixing species. Results were tested using ANOVA (one-way) Fisher's comparisons, n=5, p<0.05. Bars which share letters are not significantly different.

5.5 Conclusions

This part of the study investigated the growth response of selected native N-fixing species, and growth comparison between these N-fixing plants and a non N-fixing plant (*Pomaderris amoena*) in different soil nutrient conditions, in a pot experiment. These species responded differently to soil nutrient amendments. Higher nitrogen application rates increased growth of *P. amoena*, which was generally responsive to modified soil nutrient conditions. The four native N- fixing species (*S. microphylla, S. prostrata*, *C. australis* and *D. toumatou*) were tolerant, but generally unresponsive to elevated soil nitrogen. An exception was *C. australis* which had significantly higher biomass with the combined N / P / lime treatment. The soils with the highest application rate of N (300 kg ha^{-1}) still had elevated levels of ammonium and nitrate after 6 months growth. Nitrogen (urea) application increased soil acidity. More soil nitrate than ammonium occurred in the acid soils created by nitrogen treatments. Soil Olsen P was reduced with increasing soil pH (from 4.0-5.4). Nitrogen-fixing plants had less nitrogen uptake compared to *P. amoena* with 100 kg ha^{-1} nitrogen application. Soil nitrogen inhibited nodulation of roots but soil phosphorus promoted nodulation of the native legumes. The N-fixing plants contributed significant amounts of nitrogen to the soil, and there was evidence from the prior experiment (Chapter 4) that this would be beneficial to the growth of other native species including *Pomaderris*.

Chapter 6

Response of native N-fixers to nitrogen and phosphorus in field conditions

6.1 Introduction

In New Zealand, over 40% of the land is used for farming (pasture and cropping), and only about 30% is native forest and shrubland (Glade, 2003; Tate et al., 2003). The rest is tussock grassland, urban areas, wetlands and alpine zones (Clarkson et al., 2007; Mark & Dickinson, 2008). The South Island contains most of the farmlands, especially the Canterbury plains (Price, 1993). The fertility of natural New Zealand soils is variable but generally low, so fertilizers are widely used where plants and animals are raised (Condron et al., 2000). Nitrogen and phosphorus application has substantially increased in recent years with intensification of farming (Baskaran et al., 2009; Dynes et al., 2010; Sparling & Schipper, 2004).

Human-induced flows of nitrogen and phosphorus are now a major influence on the earth's biogeochemical cycles (Galloway et al., 2008). Positive changes have been made to these nutrient cycles that have been necessary to enhance the agricultural production to support the growing human population (Galloway & Cowling, 2002). However, there have also been negative effects, both on the natural environment and agriculture systems. Urea as a cheap and convenient source of nitrogen fertilizer is widely used in agriculture to facilitate soil nitrogen for a better growth of plants (Kennedy et al., 2004). Nitrogen fertilizer has increased 20-fold from 1900 to 2000, which is much more than biological nitrogen fixation by legumes (Bouwman et al., 2013). Even though nitrogen fertilizer usage is increasing rapidly, nitrogen fixation still plays an important role in contributing nitrogen supplements for plants and in the global nitrogen cycle (Amanullah et al., 2009). Furthermore, Zahran (1999) suggested that rhizobium-legume symbiosis could be the ideal solution for soil fertility improvement and rehabilitation. However, there is limited knowledge of the effects of fertilizer application on rhizobium-legume symbiosis in agricultural landscapes. This chapter aims to investigate these interactions in the field situation, in the context of the role of native N-fixers in restoration trajectories on agriculturally-modified soils.

In work described in this chapter, four native N-fixing plants (*S. microplylla, S. prostrata, C. australis and D. toumatou*) were planted in the Eyrewell restoration area, developing studies of a greenhouse experiment reported in Chapter 5. The earlier results showed all these native species could tolerate

300 kg ha^{-1} nitrogen application to soil. Nitrogen and phosphorus applications did not change the plant dry biomass but soil properties were modified by different plant species under different soil conditions.

6.2 Materials and Methods

6.2.1 Field site description (Eyrewell Reserve)

Eyrewell Forest (Figure 6.1) is located on the north plain of the Waimakariri River, in Canterbury (Molloy & Ives, 1972) and is the site of a current rapid conversion from pine plantation forest to dairy farmland. The original vegetation was cleared by burning by Polynesian settlers, and later converted to sheep grazing by Europeans. In this sequence, large trees were replaced by kanuka-dominated shrubland, and then largely converted to dry sheep-grazed grassland. The Eyrewell plantation forest was established in the 1930s, on land where the soil quality was poor, and previously thought to be only capable of supporting hardy grasses without introducing considerable amounts of irrigation (Papesch et al., 1997). The converted forest contains two small protected reserve areas with natural vegetation including kanuka (*Kunzea robusta*), *Pomaderris*, *Discaria* and *Carmichaelia*. At the time of the present study, a total of 17 restoration plots (amounting to a total of 150 ha) were in the process of being planted, with additional planting of native species along farm borders, paddock margins and under irrigators (Dollery, 2017).

Figure 6.1 Eyrewell Forest shown in red line frame (Imagery from Google Earth).

6.2.2 Plants and experiment design

Plants (*S. microphylla*, *S. prostrata*, *C. australis* and *D. toumatou*) were purchased from the department of Conservation Nursery at Motukarara, Canterbury. All plants had been established from seeds and were about one-year old. Plants were planted in one of the Eyrewell restoration areas (Figure 6.2, 172.316°, -43.451°). The area has not been added any fertiliser prior, and soil chemistry details of Eyrewell area shown in Appendix D.

Figure 6.2 Field plots of native N-fixing plants in Eyrewell area (Photograph, Shanshan Li). The photograph shows three plots containing a control, nitrogen and phosphorus treatments shown as 1, 2 and 3 respectively. Two meter interval exist between each plot. Other replicates are not shown in the photograph (see Figure 6.3).

Three replicates of Control, N and P plots (totally nine plots) were established (Figure 6.3). No additional nutrients were added to the Control plots. Solid urea (equivalent to 300 kg ha^{-1} nitrogen) and 470 kg ha^{-1} super phosphate were added to nitrogen and phosphorus treatment plots. Each plot contained four species (*S. microphylla*, *S. prostrata*, *C. australis* and *D. toumatou*) and 10 plants of each species. One meter interval between different species, 0.5 m between individual plants and between plant rows, and 2 m between each treatment plot (Figure 6.3). Plants were protected with

combiguards. Five of the ten plants of the three legume species (*S. microplylla, S. prostrata, C. australis*) were inoculated with rhizobia (Figure 6.3) followed planting (30ml/plant, with rhizobia information and preparation as described in #4.2.1.2). Plant arrangement and plant inoculation design is shown in Figure 6.3. Plants were planted in June, 2016, after a 7 month growth period, five of the plants were randomly harvested crossed all the replicate plots from each species and each treatment in December, 2016. The rest of the plants were kept on the restoration plot for longer-term growth. The details of air temperature and rainfall from planting to harvesting shown in Appendix D (Figure D.4).

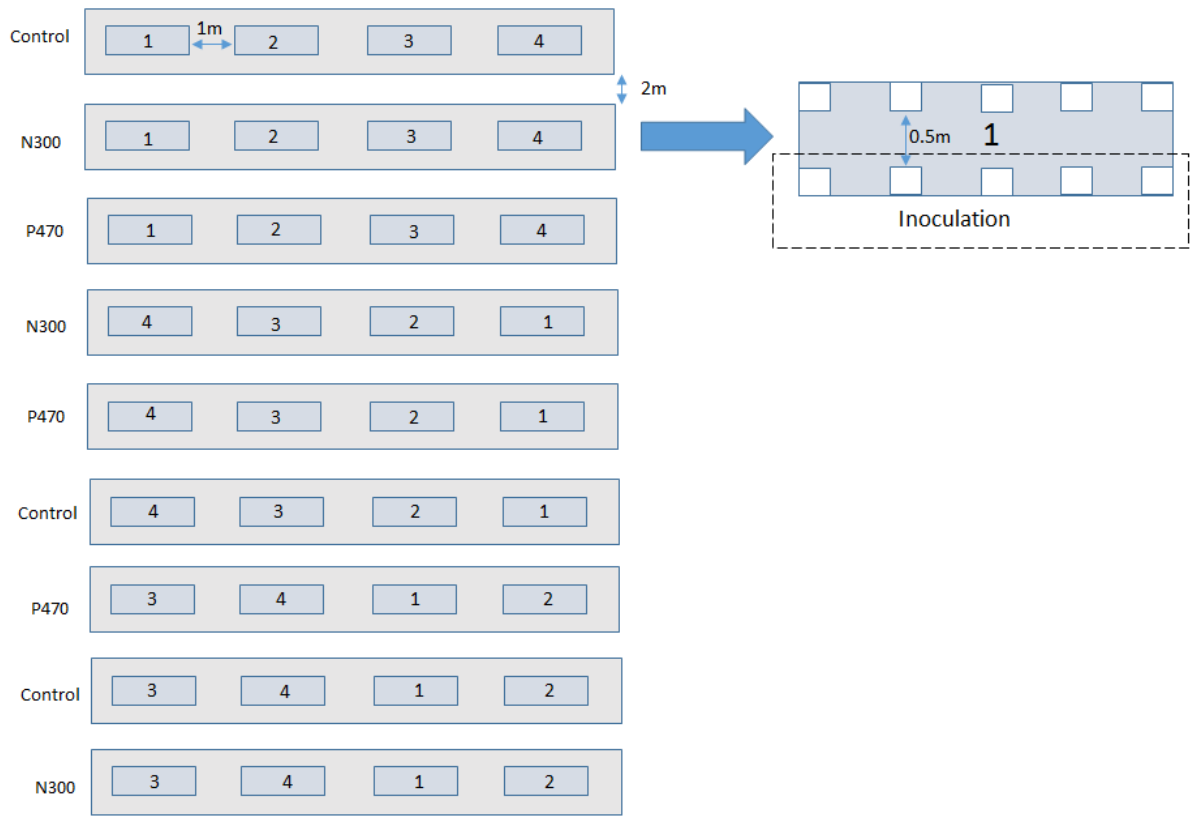

Figure 6.3 Plot design of native N-fixing plant species. Numbers 1-4 indicate 4 different plant species. "□" indicates individual plant. Control= no additional fertilizer, N300= 300 kg ha^{-1} nitrogen, P470= 470 kg ha^{-1} super phosphate. Five plants from *S. microphylla*, *S. prostrata* and *C. australis* has been inoculated. One meter interval between different species, 0.5 m between individual plantsand between rows, and 2 m between each treatment plot.

6.2.3 Plant and soil measurements

Plant height and dry weight were measured of harvested plants individually after a 7 months growth period. Plant dry biomass of above ground shoots and below ground roots were measured after being dried at 60°C for 3-5 days. Five soil samples were collected from the field (0-15cm) randomly when planting (T_0) and harvesting (T_7) for the measurement of available nitrogen contents. Soil of harvested plants collected from plant rhizosphere. Soil measurements which consisted of the concentration of ammonium and nitrate, Olsen P, and the soil pH were measured.

NH_4^+ and NO_3^- concentrations in the soil were determined following extraction with KCl. Four grams of fresh soil from each pot were transferred to 50 ml tubes, adding 40ml of 2 M KCl, then shaking for 1 hour, centrifuged at 2,000 rpm (10 mins) and filtered (Whatman 41 filter paper) following the procedure described Clough et al. (2001). All samples were analyzed by Flow Injection Analysis (FIA, Foss FIAstar 5000 triple channel, Foss Tecator, Sweden). The remaining soil was air dried for 48 to 72 hours, then ground and sieved (< 2 mm) for soil pH and total nitrogen and carbon analysis. Soil pH was measured following suspension of 5 g of dry soil in deionized water for 4 hours at the ratio of 1:5 of soil: solution (S20 SevenEasy^{TM} pH meter, Mettler-Toledo, Switzerland). For Olsen P measurement (Olsen, 1954), 1 g of dry soil was suspended into 20 ml 0.5 mol. of $NaCO_3$ in 50 ml flask. The mixture was shaken for 30 minutes then centrifuged at 2,000 rpm for 10 minutes and filtered using Whatman No. 42 filter paper. The extracted solution was added to 10 ml filtrate and two drops of p-nitrophenol. The solution was mixed with sufficient 2 M H_2SO_4 to become clear. Deionized water was mixed with 5 ml Working Colour Reagent to make up 50 ml, then shaken well and left for half an hour. The extracted solutions were analysed by a UV/VIS (UV160A) spectrophotometer (Shimadu, Japan) at 880nm.

6.2.4 Statistical analysis

Significant analysis of plant biomass, soil ammonium, soil nitrate, soil pH and soil Olsen P were tested using ANOVA's (one-way) and Fisher's comparisons (Minitab, version 17).

6.3 Results

6.3.1 Plant biomass responsed to N, P fertilizer and inoculation

Plants were protected using combiguards (as in Figure 6.2), and most of the plants survived untill harvested. However, the top part of some plants which extended out of the combiguard protection was damaged by hares. There were no significant differences in dry weight in either shoots or roots

of any species between treatments. The shoot height of *S. mircrophylla* decreased in all the treatments (Table 6.1).

Table 6.1 Mean (±SE) shoot height change, and shoot and root dry weight after harvest, of native N-fixing species. Results were tested by ANOVA (one-way) Fisher's comparison (n=5). Numbers in brackets indicate the standard error of the mean. Means which share same letters are not significantly different for each species. ** means p<0.01. Control= no additional fertilizer, N300= 300 kg ha^{-1} nitrogen, P470= 470 kg ha^{-1} super phosphate. (I) indicates plants were inoculated with rhizobia and (-) indicate plants were not inoculated.

Height increase(cm)			Species	
Treatments	*S. microphylla*	*S. prostrata*	*C. australis*	*D. toumatou*
Control(-)	-2.5 (2.94) [a]	4 (1.52) [a]	-4.33 (3.48) [a]	2.5 (0.87) [b]
N300(-)	-6.38 (7.64) [a]	-4.5 (3.47) [ab]	-11.4 (9.42) [a]	7.8 (1.79) [a]
P470(-)	-0.9 (6.93) [a]	-5 (3.02) [b]	0.9 (7.04) [a]	3.3 (0.54) [b]
Control(I)	-2.4 (1.29) [a]	-3.8 (3.72) [ab]	-8 (4.34) [a]	-
N300(I)	-3.3 (3.93) [a]	-4.17 (5.53) [ab]	-12.2 (5.48) [a]	-
P470(I)	-4.5 (2.49) [a]	-1.6 (1.81) [ab]	-6.2 (6.77) [a]	-
P value	0.972	0.319	0.767	0.001**
Dry weight(shoot)g			Species	
Treatments	*S. microphylla*	*S. prostrata*	*C. australis*	*D. toumatou*
Control(-)	5.89 (1.02)[a]	3.38 (0.46)[a]	13.07 (1.30)[ab]	2.76 (0.97)[a]
N300(-)	4.7 (0.37)[a]	3.97 (0.57)[a]	10.91 (2.31)[ab]	3.31 (0.48)[a]
P470(-)	7.10 (1.04)[a]	5.73 (1.57)[a]	14.25 (1.23)[a]	4.31 (0.92)[a]
Control(I)	8.11 (1.85)[a]	7.03 (1.19)[a]	13.15 (1.42)[ab]	-
N300(I)	5.44 (1.03)[a]	6.42 (2.99)[a]	10.16 (0.80)[b]	-
P470(I)	5.73 (0.69)[a]	7.25 (2.2)[a]	14.65 (1.33)[ab]	-
P value	0.367	0.352	0.221	0.437
Dry weight(root)g			Species	
Treatments	*S. microphylla*	*S. prostrata*	*C. australis*	*D. toumatou*
Control(-)	2.44 (0.74) [a]	2.38 (0.39) [b]	4.27 (0.27) [a]	1.53 (0.35) [a]
N300(-)	2.1 (0.23) [a]	2.86 (0.62) [ab]	4.37 (0.55) [a]	0.99 (0.06) [a]
P470(-)	2.34 (0.32) [a]	4.61 (1.54) [ab]	4.39 (1.02) [a]	2.13 (0.95) [a]
Control(I)	3.67 (0.84) [a]	4.21 (0.55) [ab]	4.22 (1.12) [a]	-
N300(I)	2.78 (0.90) [a]	3.06 (1.33) [ab]	5.29 (0.82) [a]	-
P470(I)	2.07 (0.38) [a]	5.72 (1.54) [a]	7.28 (3.00) [a]	-
P value	0.473	0.267	0.710	0.420

The shoot height of *S. prostrata* and *C. australis* increased under Control and P470 treatments respectively, but decreased in the other treatments. *D. toumatou* shoot height was enhanced in all the treatments. The height increase of *D. toumatou* under N300 treatment was higher than the Control and P470 treatments (Table 6.1, $p<0.01$), but there was no significant difference of height increase for the other three species.

6.3.2 Soil properties

6.3.2.1 Modifications of available soil N and P by plants in unfertilised soil

Unfertilised soil (without the N-fixing plants), the NH_4^+ and NO_3^- contents declined over time. The soil NO_3^- of T_7 (soil from the field when plants just planted) was significantly lower than T_0 (soil from the field when plants harvested) (Figure 6.4, $p<0.01$).After 7 months' growth period, soil with *C. australis* provided higher soil NH_4^+ than soil of T_7 (Figure 6.4, $p<0.01$). Soil with the four N-fixers provided higher NO_3^- than T_7 soil (Figure 6.4, $p<0.01$). Soil NH_4^+ of *C. australis* and *D. toumotou* was higher than *S. prostrata* (Figure 6.4, $p<0.01$). Soil NO_3^- of the two *Sophora* species were lower than *C. australis* and *D. toumotou* but higher than T_7 (Figure 6.4, $p<0.01$).

Olsen P and soil pH were not significantly different throughout the 7 months in the unfertilised soil in the field, either with native plant growth or without (Figure 6.5).

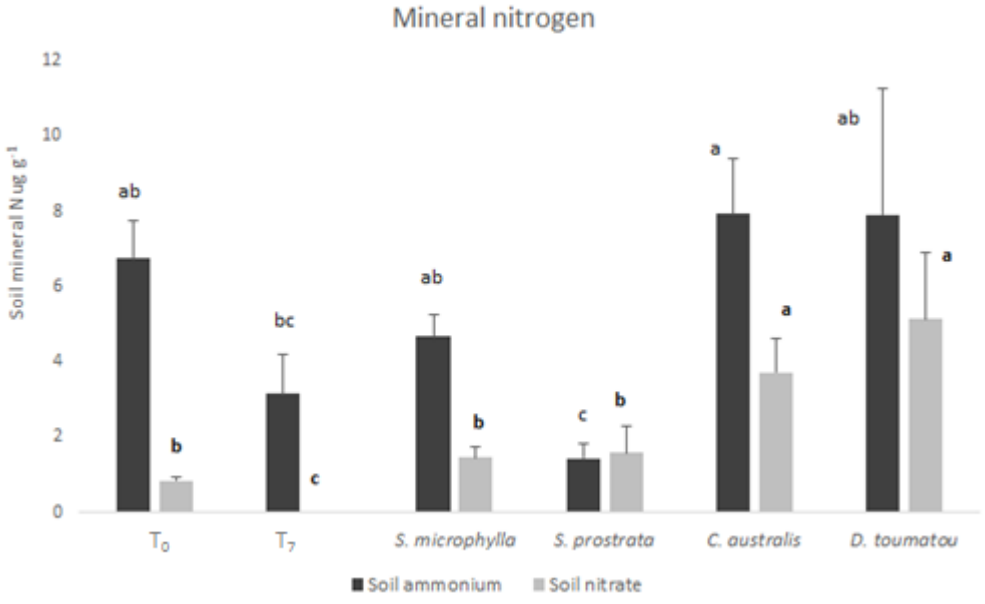

Figure 6.4 Mean (±SE) soil mineral nitrogen between different stages and species in unfertilised witout inoculation. Results were tested using ANOVA (one-way) Fisher's comparisons (n=5, $p<0.01$). T_0= soil collected when plants just planted. T_7= soil collected when plants harvested. Means which share same letters are not significantly different for soil ammonium and nitrate respectively.

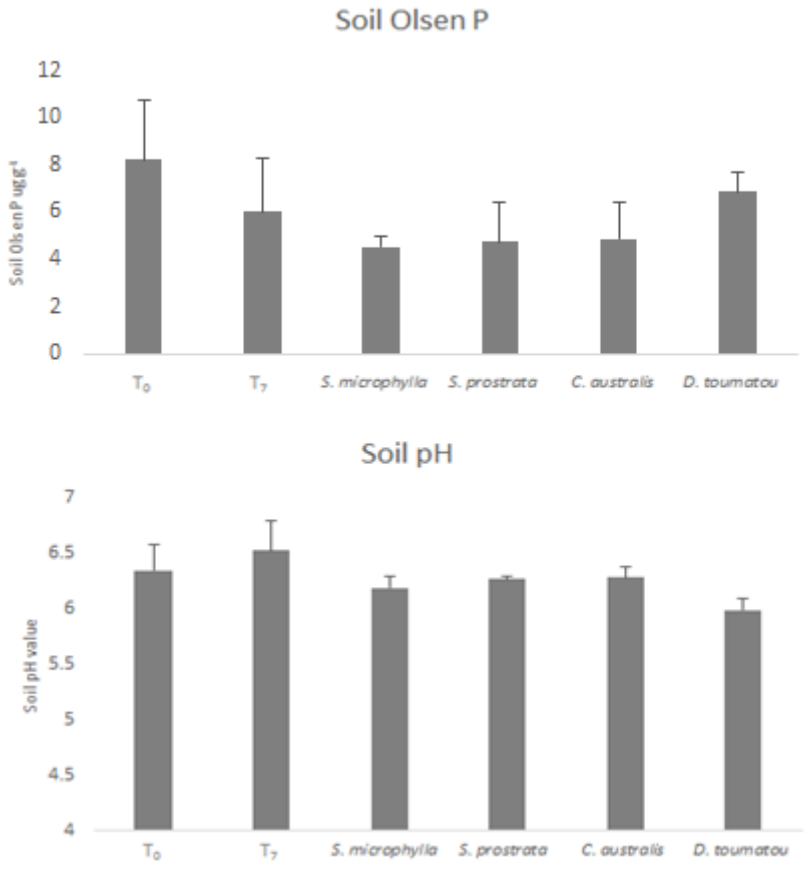

Figure 6.5 Mean (±SE) soil Olsen P and pH nitrogen between different stages and species in unfertilised without inoculation. Results were tested using ANOVA (one-way) Fisher's comparisons (n=5, p>0.05). T_0= soil collected when plants just planted. T_7= soil collected when plants harvested.

6.3.2.2 Effects of fertilizers

There was no significantly different in soil NH_4^+ under N300 and P470 treatments for any native species compared with the control group to which no fertilizer and inoculation had been added (Figure 6.6a). Comparing different species under the P470 treatment, it could be seen that the soil NH_4^+ of *C. australis* was higher than *S. prostrata* ($P<0.05$, Figure 6.6a).

Nitrogen application led to higher soil NO_3^- for the two *Sophora* spp. and *D. toumatou* (Figure 6.6b, $p<0.05$) compared to the control. This was not the case for *C. australis*. Under phosphorus treatment, *C. australis* and *D. toumatou* provided higher soil nitrate than *S. prostrata* (Table 6.6b, $p<0.05$).

As would be expected, soil Olsen P was higher with phosphorus applications compared to the control group and nitrogen treatments for the native species (Figure 6.6c, $p<0.05$). This did not appear to be the case with *S. prostrata*; soil Olsen P was higher under the phosphorus treatment

than the nitrogen treatment (Figure 6.6c, $p<0.05$), but there was no significant difference with the control.

Soil pH did not show significant differences in relation to plant species under different fertiliser treatments without inoculation (Figure 6.6d).

6.3.3.3 Impacts of inoculation on soil properties

Rhizobia had been inoculated on the three native legumes (*S. microphylla*, *S. prostrata* and *C. australis*) but there was no significant difference of soil mineral nitrogen, soil pH and soil Olsen P between the inoculation group and non-inoculation group. This applied to the control, nitrogen and phosphorus treatments for all the three species (Table 6.2). Furthermore, nodules were found in both the inoculated and non-inoculated plants under the different fertilizer applications. Native plants had more nodules with phosphorus application than the control, and with nitrogen treatments (Figure 6.7, $p<0.01$). There was no significant difference in the number of nodules between the inoculation and non-inoculation group.

Table 6.2 Mean (±SE) soil ammonium, nitrate, pH and Olsen P under Control, N300 and P470 treatments with and without inoculation of the native legume species (*S. microphylla*, *S. prostrata* and *C. australis*). Numbers in brackets indicate the standard error of the mean. Results were tested using ANOVA (one-way) Fisher's comparisons (n=15, p>0.05). Control= no additional fertilizer, N300= 300 kg ha^{-1} nitrogen, P470= 470 kg ha^{-1} super phosphate. I(+) indicates plants were inoculated with rhizobia, I(-) indicates plants were not inoculated.

	Soil property			
Treatments	Ammonium (ug g^{-1})	Nitrate (ug g^{-1})	pH	Olsen P (ug g^{-1})
Control				
I (-)	4.68 (0.93)^{a}	2.25 (0.46)^{a}	6.24 (0.04)^{a}	4.79 (0.79)^{a}
I(+)	6.89 (2.29)^{a}	2.05 (0.59)^{a}	6.30 (0.10)^{a}	3.84 (0.44)^{a}
P values	0.391	0.794	0.562	0.307
N300				
I (-)	7.88 (1.65)^{a}	12.34 (2.89)^{a}	6.28 (0.10)^{a}	4.35 (0.56)^{a}
I(+)	10.74 (1.98)^{a}	19.79 (6.25)^{a}	6.14 (0.08)^{a}	4.45 (0.67)^{a}
P values	0.285	0.292	0.300	0.902
P470				
I (-)	7.61 (2.19)^{a}	2.32 (0.71)^{a}	6.09 (0.08)^{a}	11.23 (1.25)^{a}
I(+)	6.41 (1.59)^{a}	2.14 (0.66)^{a}	6.06 (0.07)^{a}	8.70 (0.90)^{a}
P values	0.660	0.856	0.791	0.112

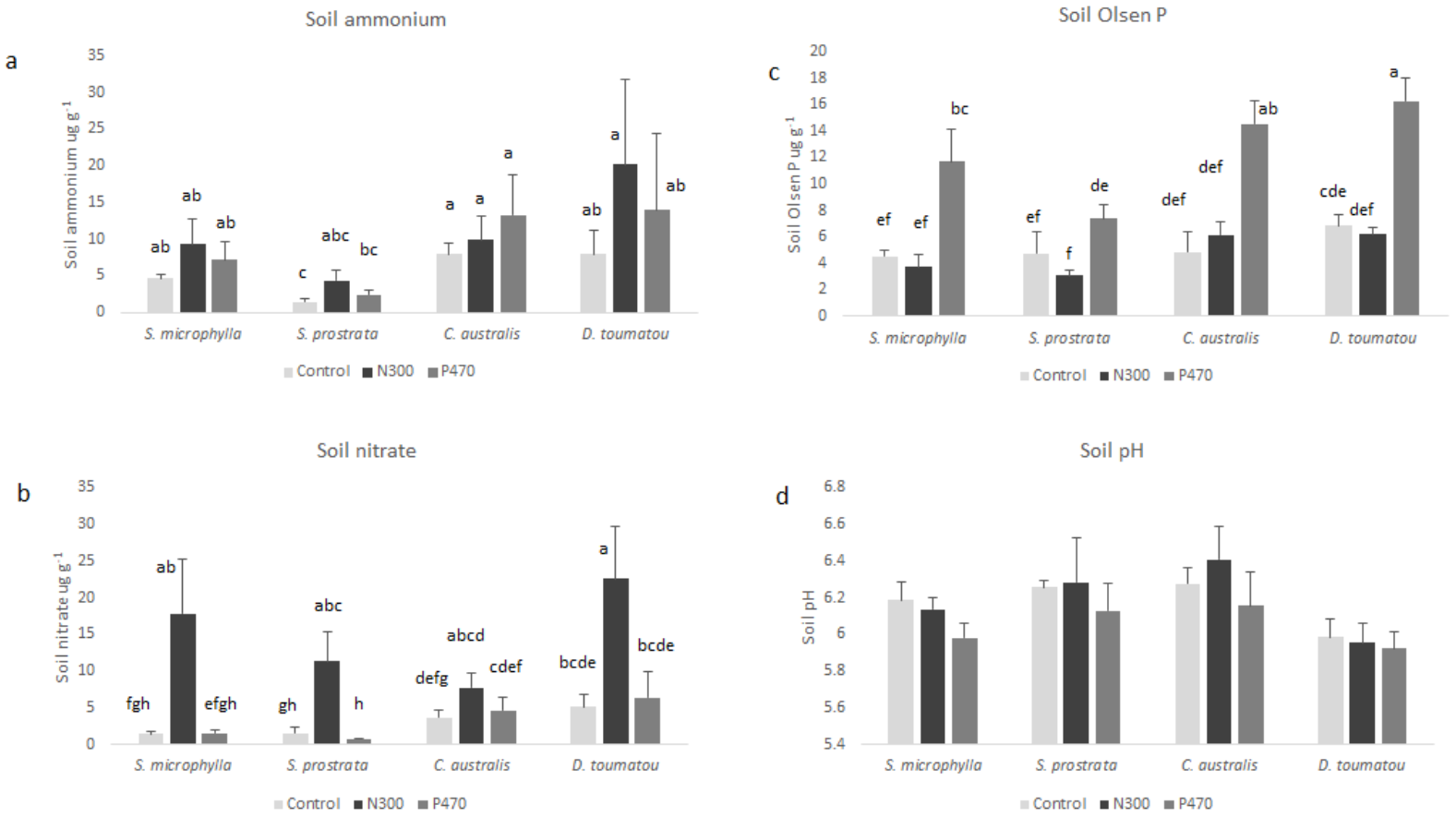

Figure 6.6 Mean (±SE) soil mineral nitrogen, soil Olsen P and pH under Control, N300 and P470 applications with the growth of the different native N-fixers without inoculation. Results were tested using ANOVA (one-way) Fisher's comparisons (n=5, $p<0.05$; soil pH $p>0.05$).

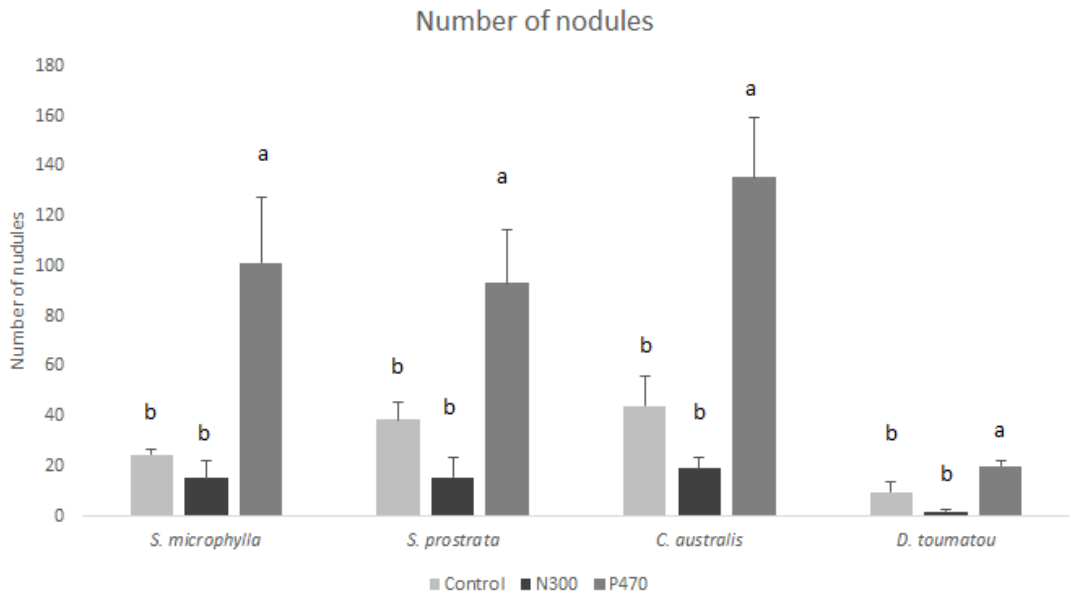

Figure 6.7 Mean (±SE) number of nodules of the native N-fixers under Control, N300 and P470 applications without inoculation. Results were tested using ANOVA (one-way) Fisher's comparisons (n=4, p<0.01). Means which share same letters are not significantly different in each species group.

6.4 Discussion

6.4.1 Plant growth, fertilizer and inoculation

Three of the legumes were damaged by hares which clearly impacted these results. However, height increased for the *D. toumatou* plant which did not extend above the combiguards and were not grazed. All plants were about one-year old but the *D. toumatou* plants were younger and smaller than the other three species. None of the species responded to nitrogen application in terms of plant dry weight. In previous studies, other species of legumes have been shown to respond to N fertilisation; for example, 60 to 70 kg N ha^{-1} contributed to yield increases of soybean (Taylor et al., 2005). In one of the few prior studies on the same species used in the present study, Franklin et al. (2015) found that application of 200 kg N ha^{-1} did not make any difference to *S. microphylla* dry biomass. It appears likely that native, N-fixing species do not respond, positively or negatively, to high levels (200-300 kg N ha^{-1}) of nitrogen applications but it is clear they can tolerate these levels of N fertilizer application. Elsewhere it has been suggested that small starter application amounts of N fertilizer may be suitable for the growth of grain legumes (Van-Kessel & Hartley, 2000).

Contrary to uncertainty about the benefits of nitrogen, phosphorus fertilizer is known to benefit nodulation, legume biomass and to promote the capability of nitrogen fixation of legumes (Besmer et al., 2003; Sanginga et al., 2001). Dry matter accumulation has been shown to respond to P application (9-18 kg ha^{-1}) although this depends on the legume species (Carsky et al., 2001). Fifty kg ha^{-1} P application was essential to produce good legume and subsequent cereal crop yield (Kihara et al., 2010). In the present study, P application (470 kg ha^{-1}) did not significantly increase plant dry biomass, but it promoted the nodulation which similarly to the prior finding in the glasshouse experiment (Chapter 5).

Similar to the finding of the greenhouse experiment in Chapter 5, the legumes (*S. microphylla*, *S. prostrata* and *C. australis*) did not significantly respond to inoculation under control, 300 kg ha^{-1} N and 470 kg ha^{-1} P treatments. All the plants which have been inoculated were about one-year old and inoculation did not significantly increased their growth in natural soil, or with N and P applications. The native legumes were not significantly responded to inoculants with 30ml/plant inoculation rate that may be due to the inoculants are ubiquitous in these soils, or the competition between with local strains. Further work would be required to determine which strains of rhizobia had colonized the roots, and how much inoculantion of effective strains are optimal for plant growth.

6.4.2 Soil status with N-fixers growth and fertilizer applicaion

Soil available N decreased naturally in the field over the 7 months' growth period, but Olsen P was not significantly different. This is because nitrogen is required in larger amounts than phosphorus, and nitrogen in soil is more mobile than phosphorus; NH_4^+ and NO_3^- are transient species of this element (Patrick Jr & DeLaune, 1976; Richardson et al., 2009; Verhoeven et al., 1996). Various factors affect biological nitrogen fixation in the field, associate with plant persistence and production, soil nitrogen status and competition with grasses (Bohlool et al., 1992; Ledgard & Steele, 1992). Nitrogen fixation and plant production are also affected by soil moisture status, soil nutrient and pH, as well as by soil fauna (Russell, 2002; Woodfield et al., 1996). *Carmichaelia australis* and *D. toumatou* provided more soil nitrate compared to the two *Sophora* spp. in the field without fertilizer applications, whereas the greenhouse experiment results (Chapter 5) indicated that *Sophora* spp. provided more soil mineral N may due to different environment conditions (light, water and temperature) effected on the nitrogen fixation of these N-fixers. In both the field and greenhouse experiments, although there was a difference in the contribution from N-fixing plants in different growth conditions, N-fixers were able to maintain or increase the status of available soil nitrogen. In the control treatment, soil Olsen P was not significant difference between the N-fixers in the field

trial, whereas they were higher with *Sophora* than *D. toumatou* in the greenhouse pot trial. This may due to the uptake of the grasses associated with the N-fixers in the field.

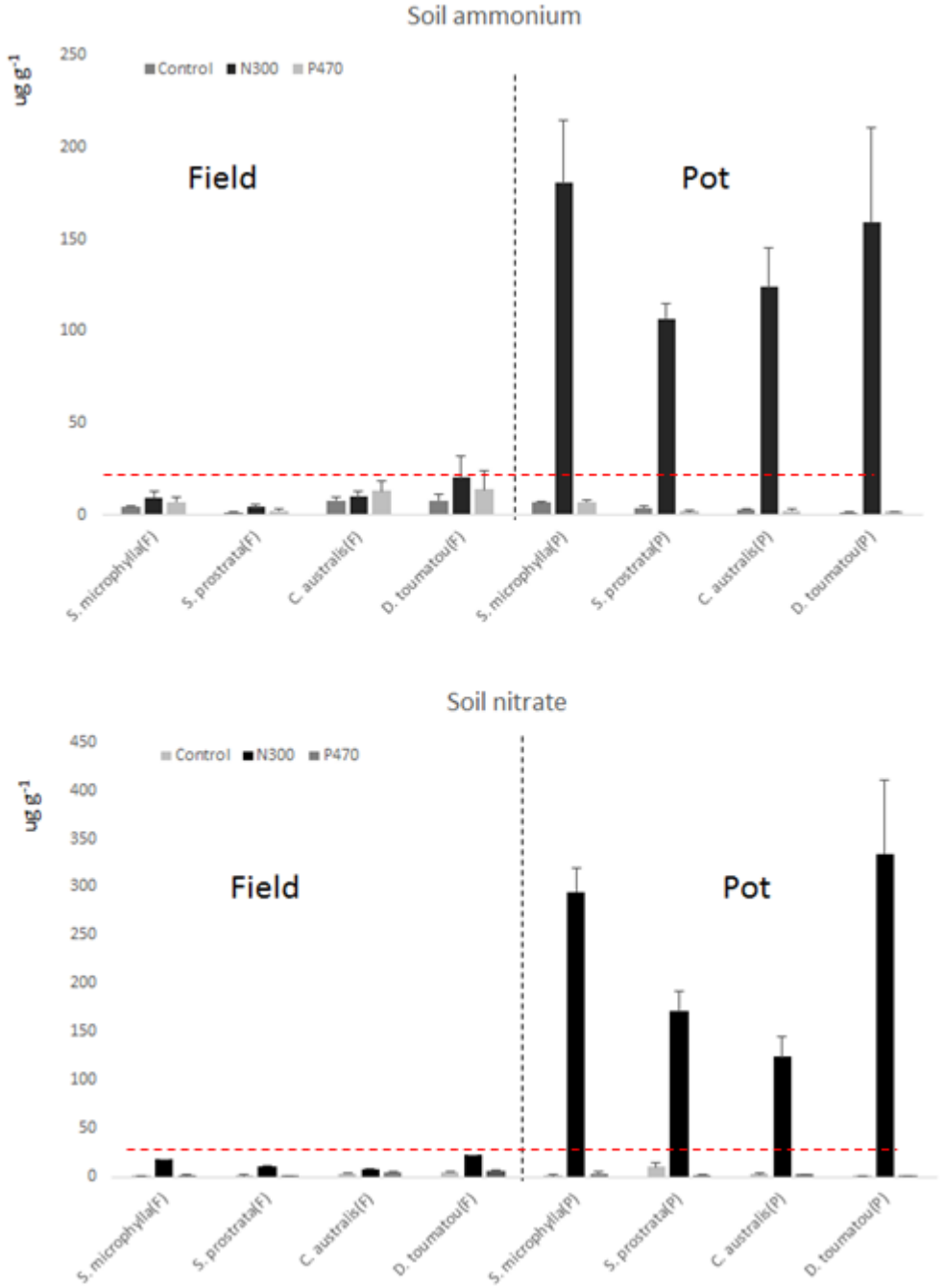

Figure 6.8 Mean (±SE) soil mineral nitrogen under control, N300 and P470 treatments with the growth of different native N-fixers without inoculation. Plants under different treatments shown as different colours of bars. (F) and (P) followed with plant species indicate these plants grown in the field and pots in greenhouse respectively.

For all the N-fixing species, soil NH_4^+ in the field was not affected by N and P fertilizer applications. Soil nitrate was significantly increased by adding N fertilizer, except with *C. australis* in the field. In glasshouse experiment, both NH_4^+ and NO_3^- were much higher ($p<0.01$) with N application (300 kg ha^{-1} N) when the plants were grown in pots in the greenhouse, compared to the field (Figure 6.8). This led to N (urea) fertilizer significantly reduced soil pH in the pots ($p<0.01$), whereas the soil pH was not changed significantly in the field (Figure 6.9). Hydrolysis and oxidization by N fertilizer caused the soil pH to decrease in the pots which was discussed previously in #5.4.2; an influx of protons accompanying uptake of NO_3^- may also change soil pH (Bowen & Rovira, 1991; Meharg & Killham, 1990). For the field location, uptake of N from the soil by neighbouring grasses has been shown to directly reduce soil N content, which then increases soil pH (Sanginga et al., 2001). This also abridged the inhibition of biological nitrogen fixation from the N-fixers (Sanginga et al., 2001), which caused nodules occur in the field even with N application.

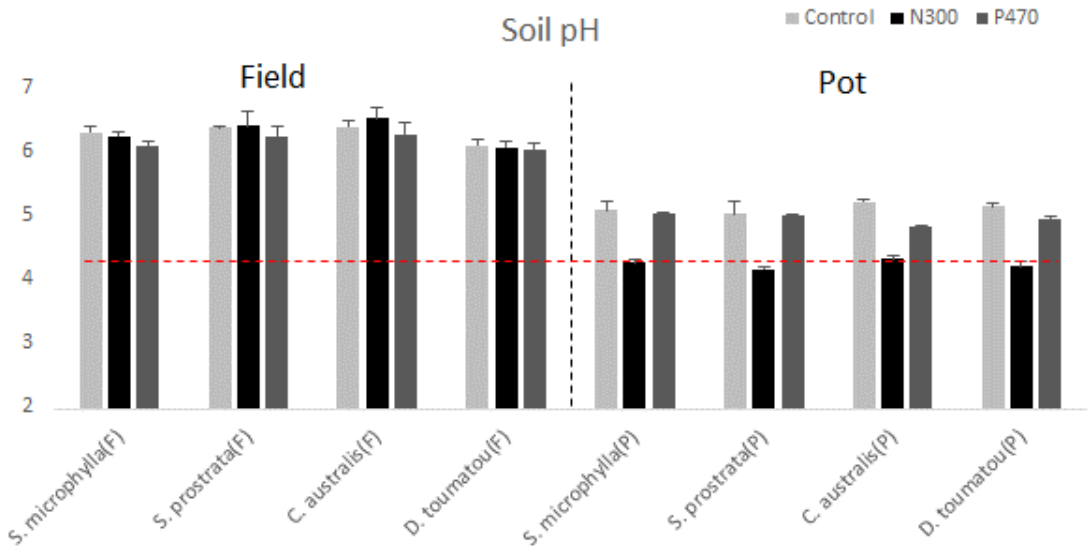

Figure 6.9 Mean (±SE) soil pH under control, N300 and P470 treatments with the growth of different native N-fixers without inoculation. Plants under different treatments shown as different colours of bars. (F) and (P) followed with plant species indicate these plants grown in the field and pots in greenhouse respectively.

6.5 Conclusioins

The work in this chapter evaluated the interaction between the growth of native N-fixers and fertiliser application in agricultural soils in the field. Native N-fixing species did not respond substantially to nitrogen and phosphorus fertilizer applications, but they could tolerate 300 kg ha^{-1} N which represents typical high levels of agricultural inputs. Soil-available N decreased naturally in the field through the growth of grass during the growth seasons, while the native N-fixers were able to maintain or increase soil mineral nitrogen. The native plants did not significantly respond to phosphorus application in terms of dry biomass, but phosphorus enhanced the number of root nodules. The native N-fixing species provided a different soil mineral N statuses between the greenhouse and the field, which may have been due to the competition from grasses; 300 kg ha^{-1} N application provided much more soil inorganic N with native N-fixers growth in pots than in the field.

Chapter 7

Native nitrogen-fixing plants in the natural landscape of Canterbury

7.1 Introduction

Many of New Zealand's plant species are endemic and are found only in New Zealand, due to a large distance from other land masses and the long period of isolation of the islands (Thomson, 2011). Canterbury is a province located of the South Island, which is an important biodiversity hotpots, largely due to the impacts of land development on its rare and threatened habitats (Williams, 2006). Since the settlement of humans, a lot of native habitats have been destroyed. Agricultural business has dramatically transformed the native landscape and led to a large loss of communities of native species including nitrogen-fixing plants. The province currently contains 25% of the threatened flowering plant species of New Zealand (Williams, 2006).

In New Zealand, there are approximately 2200 flowering plant species, but only 34 indigenous species of *Leguminosae*; only around 1.5% of all the species (Spellerberg & Given, 2004; Weir et al., 2004). Compared to invasive legume species (166 species, 7.5% of invasives) and the worldwide representation of species of *Leguminosae* (8%), the proportion of native legumes in New Zealand is small (1.6%) (Allen & Lee, 2006; Wardle, 2002; Williams et al., 2002). *Sophora* and *Carmichaelia* species are the two important native genera of the *Leguminosae* which were common in Canterbury but have suffered substantial loss of habitat following clearance for agriculture (Ewers et al., 2006; Heenan et al., 2001). Around half of all the *Carmichaelia* species in New Zealand are recorded in IUCN threatened or uncommon species lists. The genus contains one at risk *(C. williamsii*), 2 uncommon species (*C. compacta, C. appressa*), three nationally endangered species (*C. torulosa, C. stevensonii, C. muritai*), 3 nationally critical species (*C. curta, C. carmichaeliae, C. hollowayi*), 4 nationally vulnerable species (*C. kirkii*, *C. juncea, C. astonii, C. crassicaulis* subsp. *racemosa*) and 5 declining species (*C. vexillata, C. uniflora, C. nana, C.* corrugata, C. crassicaulis) (De Lange et al., 1999; Head & Given, 2001).

Sophora has eight species, and 3 of them are uncommon (*Sophora longicarinata*, *Sophora molloyi*, *Sophora fulvida*) in New Zealand. *Sophora microphylla* is a small-leaved tree, up to 25 m high that has been found throughout north and south island in New Zealand. This species commonly occurs on alluvial river terraces dunes, flood plains, lake margins and hill slopes, and grows with grey scrub communities and mixed podocarp/hardwood forests (Heenan et al., 2001). *Sophora prostrata* is a

bushy shrub/small tree which can grow up to 2 m tall, and it is hardy and able to grow well in well-drained soils. It is naturally present in the eastern part of South island in New Zealand (Cockayne, 2011). It has smaller and fewer yellow flowers than other *Sophora* species. *Carmichaelia australis* is recorded in the North Island and the east of the South Island and it occurs in different types of landscapes including grassland, shrubland, coastal and rupestrian land (Gruner, 2003).

Some native birds such as the tui, bellbird and New Zealand pigeon (kererū/kūkū/kukupa) all benefit from *S. microphylla (Moors, 1983)*. They all feast on the leaves and flowers which provide an important seasonal food for native birds. Through late winter and spring, *S. prostrata* present cluster of yellow small flowers which also benefit the native birds from nectar (Gill, 1980). *Carmichaelia australis* is able to attract birds and bees, and it non-poisonous broom to animals (Burrows, 1994). Very less is reported of the communication of these N-fixers with other plant species. *Sophora* and *Carmichaelinae* species are N-fixing plants which associate with rhizobia to fix nitrogen. Some research reported that rhizobial strains which isolated from *Carmichaelinae* species and *Sophora* species were identified as *Mesorrhizobium* based on 16S rRNA sequences analysis (Tan, 2014; Weir et al., 2004).

Species in plant communities always have interaction with other plants, animals and microorganisms (Callaway, 1997). Understanding the structure and function of natural plant communities is essential to enable us to restore vegetation. Restricted native remnants naturally occur on the Canterbury Plains, such as the Eyrewell kanuka communities (Dollery, 2017; also described in previous chapters), Riccarton Bush in Christchurch, and also in the Banks Peninsula and the Port Hills reserves (Ecroyd & Brockerhoff, 2005). Legumes are selectively grazed by animals due to their high nitrogen content and therefore are vulnerable to further loss through impacts of stock and lack of regeneration opportunities (Heenan et al., 2001). In some parts of the South Island, in particular, rabbits and hares prevent natural recruitment in all but the most inaccessible sites. Clearly, it is important to understand and protect native legumes in the Canterbury region and in New Zealand.

The focus of the present chapter is to understand the natural habitats of native N-fixing plants and associated assemblages of species that occur naturally on the Canterbury Plains. Although the growth characteristics and preferred growth conditions of many of these nitrogen-fixing species have been described, less is known of their natural species assemblages and associated soil status in natural or semi-natural conditions.

7.2 Methods

7.2.1 locations and N-fixers

Seven different locations (L1- L7, Figure 7.1) of native nitrogen-fixing species (*S. microphylla*, *S. prostrata*, *C. australis* and *D. toumatou*) were identified in different parts of Canterbury. Location 1, 2, 3 and 4 are situated in Hurunui district in northeast Canterbury (Figure 7.1) and Location 1, 2 and 3 are situated on the Mt Cass Road, with the presence of *D. toumatou, C. australis* and *S. microphylla*. Location 4 which contained *S. prostrata* located in a rocky area near to the Waipara Gorge (Table 7.1). This high country area has natural vegetation including trees and shrubland within an agriculture landscape. Location 5 is situated on the Port Hills, alongside Summit Road, between Christchurch city and Lyttelton. The area of location 5 which found of *S. prostrata* is situated in a natural shrubland on the Port Hills (Table 7.1). Location 6 is situated nearby Manaia Wildlife Habitat, on the boundary of Little River township (Figure 7.1). *Sophora microphylla* at Location 6 was found beside a stream at the edge of a forest (Table 7.1). Location 7 is about 3km from Location 6, on Mt Bossu in the east of Canterbury (Figure 7.1). *Carmichaelia australis* and *D. toumatou* were found at Location 7 which was shrubland with some trees (Table 7.1).

7.2.2 Plant record and soil sampling

In February 2017, field study areas (each 25 m^2) at each of the 7 locations were centred on the presence of targeted nitrogen-fixers. Species (including nitrogen-fixers) of trees, shrubs and ground cover in the study area were recorded. Three bulked soil samples were collected randomly using a trowel (0-15 cm depth) in each area, and stored in polythene bags. Immediately on return to the laboratory, the fresh soil was sieved through a 2mm sieve then measured for soil mineral nitrogen (NH_4^+ and NO_3^-, methods described in #4.2.1.4). The remaining soil was air dried at 25°C for 48-72 hours prior to determination of pH, total C and N (as in #4.2.1.4) and Olsen P (as in #5.2.3).

7.2.3 Statistical analysis

Differences in soil ammonium, soil nitrate, soil pH, total C, total N and Olsen P data were tested using ANOVA (one-way) with Fisher's multiple comparisons (Minitab 17), comparing each measuring area. The relationship between soil chemical properties and N-fixing plants was tested by PCA using Minitab (version 17).

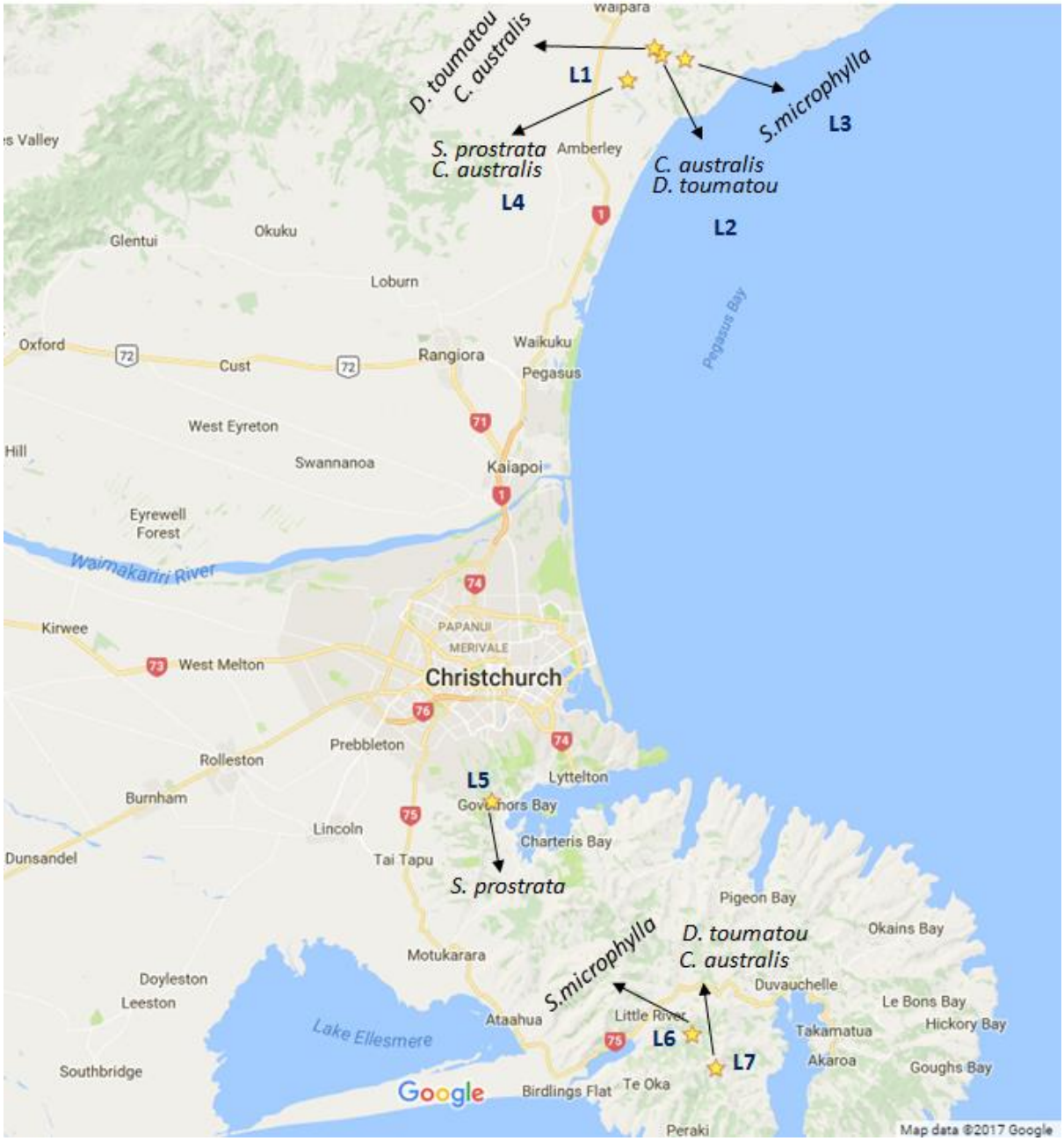

Figure 7.1 Seven Locations (L1-L7) of native nitrogen-fixing plants in Canterbury (Google map, 2017). Other numbers on the map are State Highways.

Table 7.1 Details of the locations of nitrogen-fixing species in Canterbury region. Photos were photographed by Shanshan Li and Sue McGaw in February, 2017.

Site	Location	Geographic position	N-fixers at the site	Landscape	Photos
1	Mt Cass (Hurunui district)	-43.084, 172.794	*C. australis* *D. toumatou*	Edge of forest	
2	Mt Cass (Hurunui district)	-43.089, 172.800	*C. australis* *D. toumatou*	Dry shrubland	
3	Mt Cass (Hurunui district)	-43.093, 172.824	*S. microphylla*	Forest	
4	Waipara Gorge (Hurunui district)	-43.108, 172.768	*S. prostrata* *C. australis*	Rocky area	
5	Port Hills (Between Christchurch city and Lyttelton)	-43.620, 172.633	*S. prostrata*	Hill slope	
6	Little River (East of Canterbury)	-43.786, 172.831	*S. microphylla*	Stream side forest	
7	Mt Bossu (East of Canterbury)	-43.809, 172.855	*C. australis* *D. toumatou*	Dry shrubland	

7.3 Results and Discussion

7.3.1 Native N-fixers and their surrounding plants

Four N-fixing species (*S. microphylla*, *S. ptostrata*, *C. australis* and *D. toumatou*) were found at the 7 locations (Figure 7.2). The listing of plant species at each location are presented in Appendix E.

Forty-nine different species (including 45 native species) were found to be associated with the four native N-fixing plants in the 7 different locations (within 25m^2 at each location). Location 3, with *S. microphylla*, had the most plant species (19 different species), whilst Location 4 and 5, with *S. prostrata* had the least species (5 and 6 species respectively). Different assemblages of trees, shrubs, vines, herbs, and ferns at the 7 sites reflected the different landscapes.

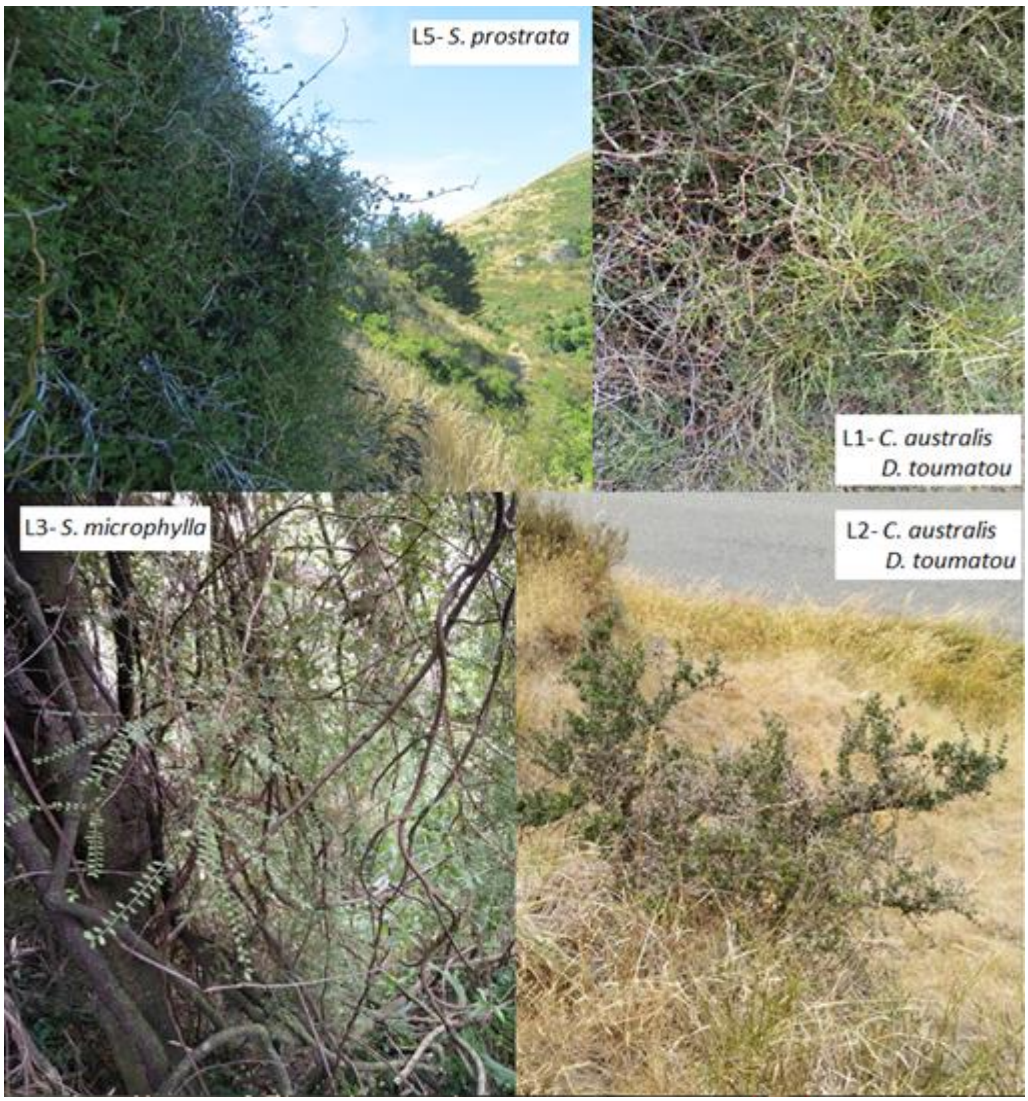

Figure 7.2 Native nitrogen-fixers (*S. prostrata*, *S. microphylla*, *C. australis* and *D. toumatou*) in Locations 1, 2, 3 and 5 (Photographed by Shanshan Li and Sue McGaw, 2017).

Carmichaelia australis* and *D. toumatou

Location 1 and 2 were hill sites that contained both *C. australis* and *D. toumatou*. *Coprosma propinqua* (a common divaricating shrub that is found in shrubland and forest margins throughout New Zealand) was abundant (Wotton, 2002). *Coprosma propinqua* was recorded in a shrubland at Location 2 and 7, and a forest margin at Location 1, growing together with the two native N-fixers (*C. australis* and *D. toumatou*).

A second common associated species was the vine *Muehlenbeckia complexa*, which is a native species are naturally found on sand dunes and coastal scrub throughout New Zealand (Greene, 1998; Wotton, 2002). This species appeared in the Locations 1 and 2, but was not found at L7 even though it is near to the coast. Assemblages of *Coprosma propinqua*, *Discaria toumatou*, and *Muehlenbeckia complexa* have previously been recorded together on the slumped and eroding slants with limestone, calcareous and glauconitic mudstones in North Otago (Molloy et al., 1999), although, in the present study, they were located in shrubland and forest margin. These three species appear naturally to grow together with *C. australis*.

Gruner (2003) stated that the distribution of *C. australis*, as a shrubland species, prominent in degraded or disturbed vegetation, such as in *D. toumatou* shrubland and *Hieracium pilosella* herbfields. *Discaria toumatou* is a spiny shrub that is important associate of short tussock grasslands and is useful in forming plantation for some areas like riverbeds and eroded slopes (Keogh & Bannister, 1992; Thomas & Spurway, 2002). The two native nitrogen-fixing species (*C. australis* and *D. toumatou*) could be used for restoration in the Canterbury high country and may be beneficial to herbal and grass establishment because they naturally stand well with various foliage and groundcover in the studying sites in Canterbury.

Sophora microphylla

Sophora microphylla grows throughout New Zealand and is described as being mostly present as mature trees within browsed riparian or forest sites (Heenan et al., 2001; Thomas & Spurway, 2002). In the present study, *S. microphylla* was similarly found at Location 3 (Mt Cass) and 6 (Little River) in Canterbury. These were mature trees, over 10 meters tall in small forest patches. Two tree species (*Melicytus ramiflorus*, *Pittosporum eugenioides*) and one vines (*Muehlenbeckia australis*) were present at the both locations. *Solanum aviculare* (a tall shrub) was also found under the *S. microphylla* trees at the two locations. *Muehlenbeckia australis* was found growing through with *S. microphylla* at the L3 and L6 indicate an association with mature *S. microphylla* trees. Some *S. microphylla* seedlings were also recorded at Location 3 (Mt Cass). It is known that the juvenile form

of *S. microphylla* has a variable duration that may take 20 years to maturity in some low areas of South Island, but juveniles have more frost resistance than adult forms (Darrow et al., 2001; Lange & Heenan, 2006). There appears to be little existing published information on species that are associated with *S. microphylla* in natural habitats. In the present study, seventeen different associated species were recorded at the two locations (L3 and L6), within a land area of $25m^2$. Herbs and ferns were only found at the L3, in the northeast of Canterbury; at L6, Little River on Banks Peninsula, there was evidence of local pest trapping at this wildlife habitat camping area that may be indicative of disturbance (grazing and trampling) impacts on the ground flora.

Sophora prostrata

Sophora prostrata was found at Location 4 (Waipara Gorge) and 5 (Port Hills) of 1.5 to 2m height with 4m canopy width. *Carmichaelia australis* was found nearby the *S. prostrata* at L4, but was not found at L5. *Sophora prostrata* tends to be found in rocky, windy and/or dry habitats in New Zealand (Thomas & Spurway, 2002; Wiser, 2001). In the present study, unlike *S. microphylla*, this species was found at a rocky area (Hurunui, L4) and on a hill slope (Port Hill, L5). These are both harsh and exposed habitats indicating a tolerance to these conditions. *Sophora prostrata* could be used for restoration in low nitrogen, dry and harsh landscapes.

Compared to *S. microphylla*, not many associated plant species were recorded with *S. prostrata* at the locations (L4 and L5). A native vine, *Muehlenbeckia complexa*, intertwined with *S. prostrata* at both L4 and L5. Two exotic species, *Echium vulgare* (viper's bugloss) and *Cytisus scoparius* (broom) grew around the *S. prostrata* at L4 and L5 respectively. *Echium vulgare* is an introduced herb which is native to Europe and temperate Asia and it has been found in dry calcareous and heath lands (Graves et al., 2010). In the present study, it was found in rocky and dry shrubland, an apparently habitat preference. *Cytisus scoparius* is a major weed attend in New Zealand; its occurrence nearby *S. prostrata* at L5 suggested that *S. prostrata* could survive alongside exotic N-fixing plants. This may contrast with, *S. microphylla* which found to be associated predominantly with native species.

7.3.2 Soil properties

Location 1 with *D. toumatou* and *C. australis* had the lowest soil moisture content of all the sites and Location 6 with mature *S. microphylla* trees had the highest (Table 7.2, $p<0.001$). In general, tall plant communities such as forest prefer moister and less free-draining soil conditions while open low plants like shrubland commonly occur with drier and harsh soils (Williams, 2006). Location 7 (shrubland at Mt Bossu) with *C. australis* and *D. toumatou* had higher soil moisture than L1 and L2 with *C. australis* and *D. toumatou* (Table 7.2, $P<0.001$), which may explain more plant species at L7.

Table 7.2 Mean of soil property from different natural sites. Numbers in brackets indicate the standard error of the mean (SEM). Results were tested using ANOVA (one-way) and Fisher's comparison (n=3). Numbers which share letters are not significantly different.

Location/ N-fixer	Soil moisture (%)	NH_4^+(ug g^{-1})	NO_3^-(ug g^{-1})	pH	Olsen P^-(ug g^{-1})	N (%)	C (%)	CN Ratio
L1/ *C. australis* and *D. toumatou*	4.89 (1.71)^{e}	4.65 (0.32)^{ab}	2.76 (1.27)^{d}	5.68 (0.14)^{cd}	3.13 (0.16)^{de}	0.31 (0.03)^{c}	3.76 (0.63)^{de}	12.04 (0.79)^{bcd}
L2/ *C. australis* and *D. toumatou*	9.75 (0.54)^{d}	6.30 (3.53)^{ab}	0.92 (0.13)^{d}	5.89 (0.09)^{c}	1.43 (0.28)^{f}	0.16 (0.02)^{d}	2.51 (0.26)^{e}	15.45 (0.15)^{a}
L3/ *S. mrcrophylla*	17.53 (1.51)^{b}	2.38 (2.26)^{b}	15.19 (6.13)^{ab}	6.60 (0.18)^{b}	25.20 (2.33)^{a}	0.39 (0.03)^{c}	5.12 (0.17)^{cd}	13.01 (0.76)^{bc}
L4/ *S. prostrata* and *C. australis*	12.85 (0.91)^{cd}	9.12 (4.85)^{a}	20.22 (2.85)^{a}	7.46 (0.04)^{a}	14.26 (3.65)^{b}	1.19 (0.14)^{a}	13.18 (0.21)^{a}	11.37 (1.13)^{cd}
L5/ *S. prostrata*	14.39 (0.87)^{bc}	4.26 (1.56)^{ab}	6.47 (0.49)^{cd}	5.37 (0.18)^{d}	2.48 (0.36)^{e}	0.56 (0.07)^{b}	6.37 (0.89)^{bc}	11.33 (0.25)^{cd}
L6/ *S. mrcrophylla*	22.65 (1.35)^{a}	1.51 (0.70)^{b}	11.42 (2.43)^{bc}	5.98 (0.13)^{c}	8.54 (1.29)^{c}	0.62 (0.06)^{b}	7.05 (0.89)^{b}	11.26 (0.36)^{d}
L7/ *C. australis* and *D. toumatou*	17.11 (0.91)^{b}	1.84 (0.27)^{ab}	0.89 (0.37)^{d}	5.32 (0.04)^{d}	4.45 (0.03)^{d}	0.37 (0.01)^{c}	4.96 (0.11)^{cd}	13.57 (0.36)^{b}
P value	<0.001	0.165	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001

Soil from L4 had the highest soil concentrations of total nitrogen and carbon and soil colour was the darkest of all the soils. Soils that dark brown or black in colour generally contain higher organic matter and total carbon (Brady & Weil, 2010). Organic matter mainly comes from plant and animal residues by decomposition (Goyal et al., 1999; Trumbore, 2000). This soil supported *S. prostrata* and *C. australis* growing beside rock. Soils which had more total soil carbon (at L3, L6 and L5 associated with *Sophora*s) were darker than those from L1, L2 and L7 (associate with *C. australis* and *D. toumatou*). Soil ammonium was not significantly different between locations, whilst soil total N and nitrate differed. Total N and soil nitrate contents from L3 and L6 (associated with *S. microphylla*), and L4 (*S. prostrata* and *C. australis*) were higher than L1, L2 and L7 (associated with *C. australis* and *D. toumatou*, $p<0.001$). Thus, soils collected from the locations with *Sophora* spp. had more soil nitrate than the locations with *C. australis* and *D. toumatou*. This could be due to either or both N mineralization from organic matter (Crohn, 2004) or the contribution of N fixation from the two *Sophora* spp..

Principal component analysis (PCA) of soil properties

Soils from different sampling locations were separated through PCA multivariate analyses in terms of soil chemical characteristics (Figure 7.3). The first component was associated with increasing soil nitrate, total soil nitrogen and carbon, and the second with increasing soil moisture and decreasing soil ammonium. Soil from L4 with *S. prostrata* and *C. australis* were related with soil nitrogen and carbon, which contained more organic matter than the other measuring areas. Soil moisture separated L3 and L6 with *S. microphylla*; clearly mature trees of *S. microphylla* in forest communities are at sites with higher soil moisture.

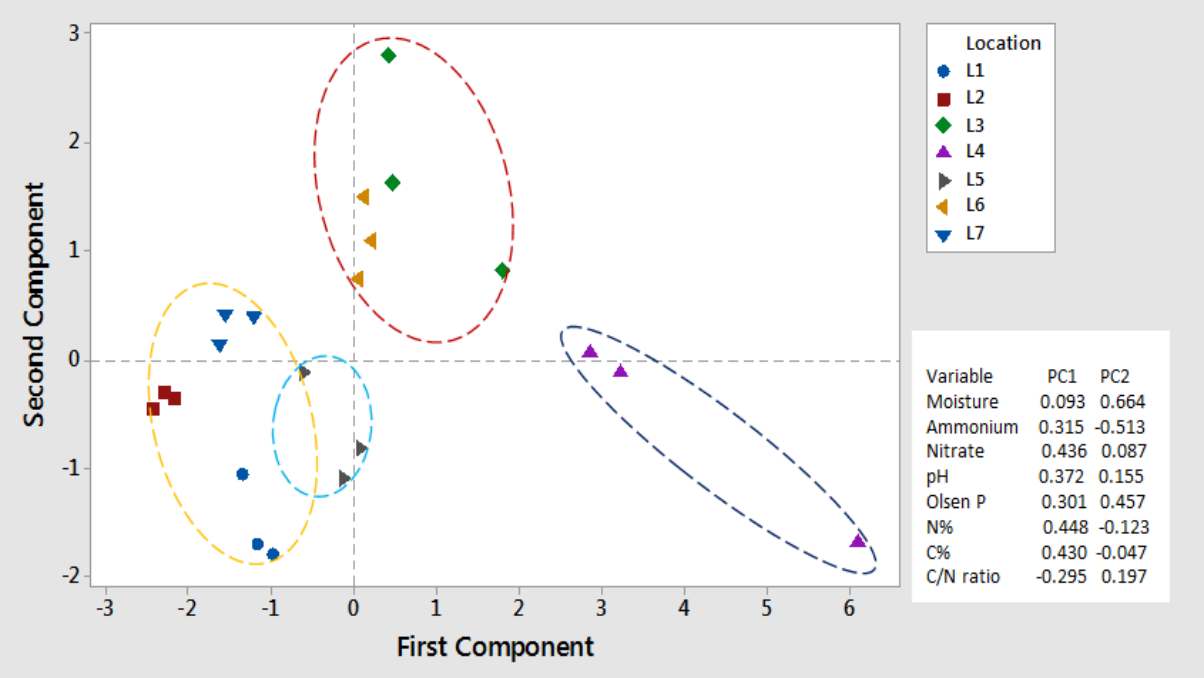

Figure 7.3 PCA analysis of surface soil chemical variables from the areas of N-fixing plants in Canterbury. Symbols in colours indicate soil samples from different locations (L1-L7). Eigenvalues of the first and second component were 4.29 and 1.39 respectively.

7.4 Conclusions

The work reported in this chapter studied plant communities and soil properties at locations where native N-fixers are naturally found in Canterbury. Four native N-fixers (*Sophora microphylla*, *Sophora prostrata*, *Carmichaelia australis* and *Discaria toumotou*) were investigated. A total of 49 species were found growing in association with these N-fixers, and most of the species in the immediate vicinity of the N-fixers were also native species (45 species). *C. australis* and *D. toumatou* were found and associated with the dry high country at Canterbury. *Sophora microphylla* was recorded in forest sites with higher soil moisture, nitrate and Olsen P than the sites of *C. australis* and *D. toumatou*. Soil ammonium was not significantly different between locations, but soil nitrate at the locations associated with *Sophora* spp. was higher than at locations with *C. australis* and *D. toumatou*; this may be related to the different amount of N contributed from N mineralization and N-fixation of the N-fixers. These findings indicate that these native N-fixing plants are an important component of diversity in natural plant communities. Their distribution is primarily determined by climatic and edaphic factors, but they appear to have a role in the structure and function of nature plant assemblages. This should be reflected in the trajectories of ecological restoration.

Chapter 8

Discussion and conclusions

8.1 Bacteria isolated from N-fixing species

Objective 1 (Chapter 3)- Isolating N-fixing related bacteria from exotic legumes (*Securigera varia*, *Astragalus cice*r and *Cytisus proliferus*) with identification.

This objective was put in place to establish and practice methodologies and the rationale for their use in the broader onward research project. In the process new knowledge was obtained relating to nitrogen symbiosis in New Zealand. *Securigera varia*, *Astragalus cicer* and *Cytisus proliferus* are legumes that are exotic but have been introduced and become established in New Zealand. Little work appears to have been previously carried out on the identification of rhizobia associate with these species in New Zealand. The present study isolated and identified N-fixing bacteria associated with these N-fixers. DNA sequencing and phylogenetic analysis were used for the identification based on 16S rRNA, *recA* and *nifH* genes. *Mesorhizobium* and *Rhizobium* have previously been reported to be associated with *Securigera varia* to fix nitrogen in China and Belgium (De Meyer et al., 2009; Yang et al., 2013), and a number of *Mesorhizobium* spp. have been identified from a symbiosis with some *Astragalus* species (such as *A. sinicus* and *A. cicer*) in Asia and Europe (Gao et al., 2004; Nuswantara et al., 1999; Tan et al., 2009). The present study is the first report of *Mesorhizobium* spp. isolated from both *Securigera varia* and *Astragalus cicer* in New Zealand, and they were able to infect and form nodules with the two N-fixing species.

Bradyrhizobium is known to be effective to introduced N-fixers in New Zealand including *Acacia*, *Cytisus* and *Ulex* (Liu, 2014; Weir et al., 2004). *Bradyrhizobium* spp. were isolated from *Cytisus proliferus* in this work, supporting the finding of prior studies. Additionally, *Ochrobactrum spp.* were isolated from *Astragalus cicer* and *Cytisus proliferus*, which is also a first report in New Zealand. *Ochrobactrum* is a gram-negative genus belongs to the Brucellaceae family (Holmes et al., 1988) and they are not well known for their nitrogen fixation capability. However, the *Ochrobactrum spp.* obtained from the present work all contained *nifH* gene (which encode the nitrogenase iron protein H), and they were shown to be able to form nodules.

8.2 The influence of inoculation to N-fixers, and competition with other plant

This section evaluates the influence of N-fixing bacteria on native and exotic legumes, and interactions with *P. amoena* (objective 2 - Chapter 4), and the influence of inoculation on native legumes (integrating the findings of Capters 5 and 6).

Inoculants used in the present study promoted nodulation and soil available nitrogen of some tested species. Inoculants (for native legumes) used in Chapter 4 were previously tested on germinated seedlings and it had been confirmed they were effective in promoting plant biomass in a prior study (Tan, 2014). In the present work, however, inoculation of these rhizobia did not affect biomass acquisition of one-year old native plants in sterilized soil. Nonetheless, inoculation of one native species (*Sophora prostrata*) did increase soil available nitrogen. Inoculants isolated from exotic species and applied to their hosts in sterilized soil (Chapter 3), promoted nodulation and increased plant biomass of smaller plants (3 months old) of two exotic legumes (*Astragalus cicer* and *Cytisus proliferus*). When these native and exotic legumes grew in association with *P. amoena* (a non N-fixer) in pots, plant height and dry biomass of *P. amoena* was greater with native N-fixers than with exotic N-fixers. The most likely reason was considered to be due to the effects of increased competition from exotic N-fixers for some combination of nutrients, water and light. This is similar to the findings from Craine and Dybzinski (2013) who found competition between species has a significant role. It is possible, but less likely, that native legumes may provide more soil available nitrogen than exotics to the neighbouring plants. Additional findings of the present work indicated that planting N-fixers with other native plants increases total carbon and C:N ratio of soil. This knowledge is of potential value to soil restoration practice, suggesting companion planting of native N-fixers is beneficial.

Inoculants were applied to one-year old native plants in both a greenhouse pot experiment (Chapter 5) and in field conditions (Chapter 6). There was no significant effect on plant biomass, supporting the prior study in Chapter 4. In these latter experiments, soils were not sterilized and so this may have been caused by competition between introduced strains and local strains of rhizobia in the existing soil populations; the local strains may dominate most of the infection sites as has been reported previously (Singleton & Tavares, 1986). However, there could be other explanations; for example, phosphorus application in both the greenhouse and field experiments promoted the nodulation of native plants, and some of the N-fixers in the greenhouse experiment also had higher biomass when receiving a combination nutrients of nitrogen, lime and phosphorus when inoculated. In the field trial, competition also occurred between grasses and N-fixers for nutrients, water and

light, similar to that described above; and N-fixers may also provide some nitrogen to their neighbouring grasses. A diagrammatic illustration of my interpretation of these findings is shown in Figure 8.1.

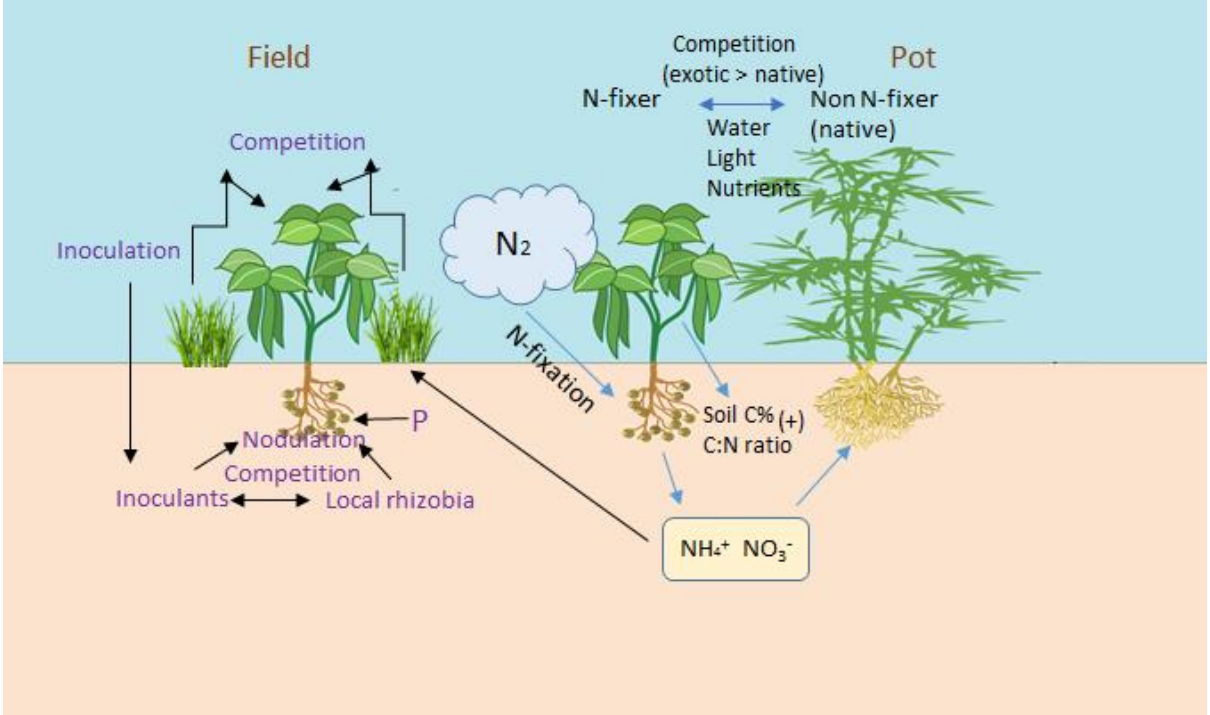

Figure 8.1 Interpretation of the findings of the present study relating to the interactions between inoculation, N-fixers and neighbouring plants.

8.3 Interaction between N-fixers and soil nutrients

This section evaluates the growth response of native N-fixing plants to different soil nutrients in glasshouse and field conditions regarding the integrated findings of Chapters 5 and 6 (Objectives 3 and 4).

The findings reported in Chapter 5 and 6 showed significant benefits of native nitrogen-fixing plants to increase soil nitrogen. Nitrogen and phosphorus were applied to native N-fixers in both glasshouse and field experiments. In the greenhouse pot experiment, nitrogen (urea) additions increase soil mineral N through microbial oxidization and nitrification (Kowalchuk & Stephen, 2001). Nitrification then releases hydrogen ions that reduced soil pH. Meanwhile, increased soil mineral nitrogen inhibits nitrogen fixation by N-fixers due to the N-fixers directly take nitrogen from the soil rather than fixing nitrogen through nodules. Since phosphorus is an essential requirement for nodulation (Qiao et al., 2007; Tang et al., 2001), the reduction of nodulation possibly also

accompany with decreases P utilization of the N-fixers. In the field trial, N fertilizer application enhanced soil NH_4^+ and NO_3^-, and it was thought that associated N uptake by grasses led to a reduction of the available N pool, which indirectly promoted N-fixation (nodulation) of the N-fixing plants. Phosphorus application significantly increased the nodulation of the N-fixers in both greenhouse and field experiments. A diagram is shown in Figure 8.2 that represents the interaction between fertilizer applications (nitrogen and phosphorus) and N-fixing plants in terms of the findings from this research.

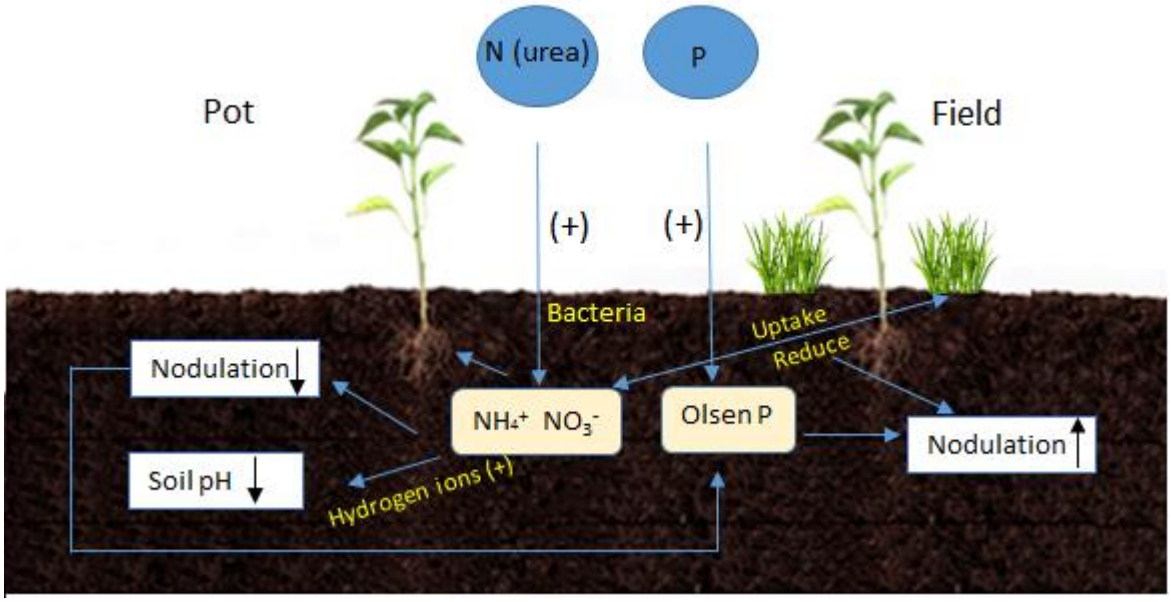

Figure 8.2 Interpretation of the findings of the present study relating to the interaction between soil fertility and N-fixing plants.

Soil pH dropped from 5.0 to 4.0 when urea was applied in the glasshouse experiment; soil with a pH of 4.8–5.2 generally restricts plant growth (Gillingham, 2017). However, in the present study, decreased soil pH did not inhibit the biomass of the native N-fixers. The native plant species and N-fixing bacteria associated with the native N-fixers appeared to be tolerant to acid soil, supporting similar findings previously reported in New Zealand (Franklin, 2014; Liu, 2014). Lime was applied only in greenhouse pot experiment, it increased soil pH but not significantly with the growth of the N-fixers. Fertilizer applications of 100 kg N ha^{-1}, with the non N-fixer (*P. amoena*) led to a lower soil nitrate status, unlike the N-fixing species. Nitrogen addition significantly promoted the biomass of the *P. amoena*. The different native N-fixing species were found to be able to maintain or enhance available N concentrations in natural infertile soils (used in the control groups) in both glasshouse and field experiments. An interesting finding from this work was that the native N-fixing plant also grew well in fertile agricultural soil, although only one native broom grew better with increased

fertility. This indicated that native N-fixers are likely to be a valuable component of plant communities in restoration in agricultural landscapes. Exotic N-fixing plants were not included in this part of the study but *Cytisus scoparius* (Scotch broom) and *Ulex europaeus* (European Gorse) present major weed problems across New Zealand and at the experimental site; they are very invasive compared to native species, but these is an evidence from the present research that native N-fixers can survive with exotic N-fixers in natural landscapes. Exotic species present at the field site are shown in Appendix D.

8.4 Native N-fixers in nature and recommendation for revegetation

This section evaluates the study of community associations and soils at locations where native N-fixers are naturally found in Canterbury (Objective 5 - Chapter 7), and considers whether recommendations can be derived from the work for practical restoration and revegetation projects.

Two *Sophora* (*S. microphylla* and *S. prostrata*), *C. australis* and *D. toumotou* species were found in natural landscapes in Canterbury that appeared to have been relatively little modified by human impacts. *Sophora microphylla* was naturally found in forest communities, and *Sophora prostrata* was found at rocky site and hill slope shrubland. *Carmichaelia australis* and *D. toumatou* were found at edges of forest and shrubland on hills. Clearly this range of N-fixers is naturally established in different landscapes, and the associated plant communities were at least partly dependent on their morphological and ecological adaptation to local conditions. The results supported the findings of glasshouse pot and field experiments, showing that they are able to grow well in soils either fertile or deficient in nitrogen and phosphorus, as well in soils with low pH; in natural landscapes, these native N-fixers were also found in various soils with different nutrient levels. Soils supporting *Sophora* spp. contained more nitrogen than those associated with *C. australis* and *D. toumatou*.

In restoration projects, native N-fixers are obviously likely to be most valuable in nitrogen degraded landscapes. In the present study, the native N-fixers studied were shown to be able to enhance soil available nitrogen and tolerate soil acidity, and to promote the growth of other native plants. This indicated that using these native N-fixing plants may be beneficial both to restoring and sustaining native plant communities. It is also likely they have a valuable role in creating healthy soils. Other work by former students in the same research group has shown that assemblages of the diverse earthworm communities are dependent on the presence of native plants (Kim et al., 2017). Other complementary research in the goup has revealed that leaf litter of native species modifies soil chemistry (Zhong et al., 2017), there are complex interactions between soil biota and soil

biogeochemistry in the rhizosphere of native plants (Kim et al., 2017), and that significant interactions native plants, earthworms and phosphorus speciation (Zhong et al., 2017).

8.5 Suggestion of further research

For further study, it would be valuable to investigate mechanisms of the interaction between N-fixing bacteria and native N-fixing plants. Real-time polymerase chain reaction could monitor the amplification of a targeted DNA during the PCR, and also could be used for detecting the amount of DNA molecules. This might be helpful for determining the efficiency of N-fixing bacteria such as measuring different N-fixing and nodulation genes in different plant tissues. N-fixing related bacteria are critical for biological nitrogen fixation. In further studies, isotope N could be used to trace how much nitrogen is fixed by different strains of N-fixing bacteria and how much nitrogen could transfer to the soil from N-fixers to neighbouring plants. Further study could also focus on the effects of N-fixing bacteria on different nodulation stages, which may inoculate bacteria at different ages of the plant seedlings (including seed). Of course, the functionality of the symbiosis is important, and it would be desirable to investigate the N-fixing capability of *Ochrobactrum* spp. in further work. These studies might reveal the specificity of communities of N-fixing bacteria to native N-fixing plants.

One limitation of this study is that only two levels of nitrogen and one concentration of phosphorus were applied to native plants. Further work could focus on clarifying the relationship between different soil nitrogen and phosphorus concentrations and native N-fixing plants. The native N-fixers were shown to have promoted the growth of a native non N-fixer in the present study. Further study comparing native N-fixing plants with exotics would be especially useful, in view of the relatively poor invasive capacity of the native species. Additionally, it would be helpful for further study to investigate the mechanisms of interaction between N-fixers and associated plants using Isotope N to trace the nitrogen flow from N-fixers to their neighbouring plants.

8.6 Overall Conclusions

This research investigated the interaction between N-fixing bacteria, N-fixing plants and soil properties, and the role of native N-fixers to restoration in agriculture landscapes. The study has shown that inoculation of N-fixing bacteria on legumes improved plant growth and nodulation but this depended on plant age and plant species. Nitrogen (urea) fertilizer application increased soil available nitrogen and led to increased soil acidity. Native N-fixing species were tolerant but not responsive to acidic high nitrogen soils. Phosphorus application improved nodulation of native N-fixers. Native legumes are able to improve the growth of other native plants, and maintain or increase available nitrogen in soil. Native N-fixers are naturally found within plant communities with

a large number of native species; 45 companion native species were recorded in the field study. These findings indicate that N-fixing plants should be considered as an essential component of the restoration matrix in the ecologically-degraded landscapes, including on fertile agriculture soils.

Appendix A

Composition of YMA and YMB media

Table A. 1 Composition of YMA and YMB (Tan, 2014)

	Contents	Weight (per litre)
YMB	Yeast extract	1.0 g
	Mannitol	10.0 g
	Dipotassium phosphate (K_2HPO_4)	0.5 g
	Magnesium sulphate ($MgSO_4$)	0.2 g
	Sodium chloride (NaCl)	0.1 g
YMA	YMB	as described
	Calcium carbonate ($CaCO_3$)	1.0 g
	Agar	15.0 g

Appendix B

Isolation from nodules of *Discaria toumatou*

B.1 Introduction

Discaria toumatou is non legume N-fixing plant native to New Zealand (Webb, 1985). It is distributed in the North Island and the South Island but very uncommon in the North Island (Wardle, 1991). It is known as a N-fixer associated with *Frankia* to fix nitrogen through functional root nodules (Newcomb & Pankhurst, 1982). Few work has been done for the isolation of *Frankia* from *Discaria toumatou* (Benson et al., 1996) due to the difficulty to isolate and grow in laboratory media (Igual et al., 2003), and there is very less known for what other bacteria could also associate with *Discaria toumatou*.

B.2 Materials and methods

Nodules collection and strain isolation

Two plants were collected from a field site alongside SH73 near Castlehead, two kilometers east of Lake Lyndon in Canterbury (-43.297, 171.741). Roots with nodules were washed in sterilized water. Ten nodules from the two plant roots and then immersed in 96% ethanol for 5 to 10 seconds in a laminar flow cabinet and transferred into 1% sodium hypochlorite solution for 3 minutes for surface sterilization followed by a sterilized water rinse several times. The nodules were then dissected by scalpel and the isolates from the nodules were streaked onto solid Defined Propionate Minimal Medium (DPM) (Baker & O'Keefe, 1984) in a Petri dish using inoculating loops. The Petri dishes were stored in an incubator at 28°C for 4 to 6 weeks in the dark. The composition of DPM is shown as Table B.1.

Table B.1 Composition of DPM (Baker, & O'Keefe, 1984)

Component	Per liter
NaPropionate	1.2 g
KH_2PO_4	1.0 g
$MgSO_4.7H_2O$	0.1 g
$CaCl_2.2H_2O$	0.01g
$FeSO_4.7H_2O$/EDTA stock	1.0 ml
Hoagland's microelements	1.0 ml
Agar(Solid medium)	15g

(Adjust pH to 6.5)

1000X Stock Iron :

Dissolve 0.75 g $Na_2EDTA.2H_2O$, 0.56 g $FeSO_4.7H_2O$ (make up to 100 ml).

Hoagland's Microelements:

Component	Per liter
H_3BO_4	2.8 g
$MnCl_2.4H_2O$	1.8 g
$ZnSO_4.7H_2O$	0.2 g
$CuSO_4.5H_2O$	0.1 g
$NaMoO_4$	0.025 g

Purification and DNA extraction

Single colonies with white radial growth of mycelium were selected from the original Petri dishes then sub-cultured onto new DPM plates, repeating this step 2-3 times to obtain a pure culture. Pure cultures were stored as mycelia suspended in DPM media. Pure strains from single colonies were inoculated in 800ul DPM media for DNA extraction.

DNA was extracted by following procedure which designed in this study. In this process 1.5 µl of lytic enzyme solution was added into 100 µl of DPM solution in 1.7 ml tubes containing mycelia cells from each isolated strain and these were then incubated at 37°C for 30 min. After 3 cycles of freezing with liquid nitrogen and thawing at 65°C, the solution was centrifuged at 1,300 *g* for 2 min. After cell collection, the extraction process was followed by QIAGEN Quick-Star Protocol (QIAGEN, 2012) using a DNeasy Plant Mini Kit.

PCR amplification and 16S rRNA analyses

The technique of polymerase chain reaction (PCR) was used to amplify the 16S ribosomal RNA gene using primers fD1 (5'- CCG AAT TCG TCG ACA ACA GAG TTT GAT CCT GGC TCA G -3') and rD1 (5'- CCC GGG ATC CAA GCT TAA GGA GGT GAT CCA GCC-3') (Weisburg et al., 1991). For the PCR recipe refer to the section 2.2.3 with the following amplification conditions: 94 °C for 1 minute 30 seconds, followed by 35 cycles of 94°C for 40 seconds, 45 °C for 45 seconds, then 72 °C for 1 minute, followed by 72°C for 7 min, 4°C for storage. PCR product gel electrophoresis and DNA analyses were as described in section 2.2.4.

B.3 Results and discussion

Phylogenetic analyses of 16S rRNA of the isolate from *Discaria toumatou*

The isolate from *Discaria toumatou* root nodules was identified as *Streptomyces* sp. by the 16S rRNA gene sequence (Figure B.1). This isolate (325 bp) was most similar to *Streptomyces bottropensis* AMCC 400023 (99% similarity, 1519 bp). Other selected *Actinobacterium* spp. were in the same group with isolate D2 and other *Streptomyces* spp.. Two selected *Frankia* spp. were separated from the group containing isolate D2.

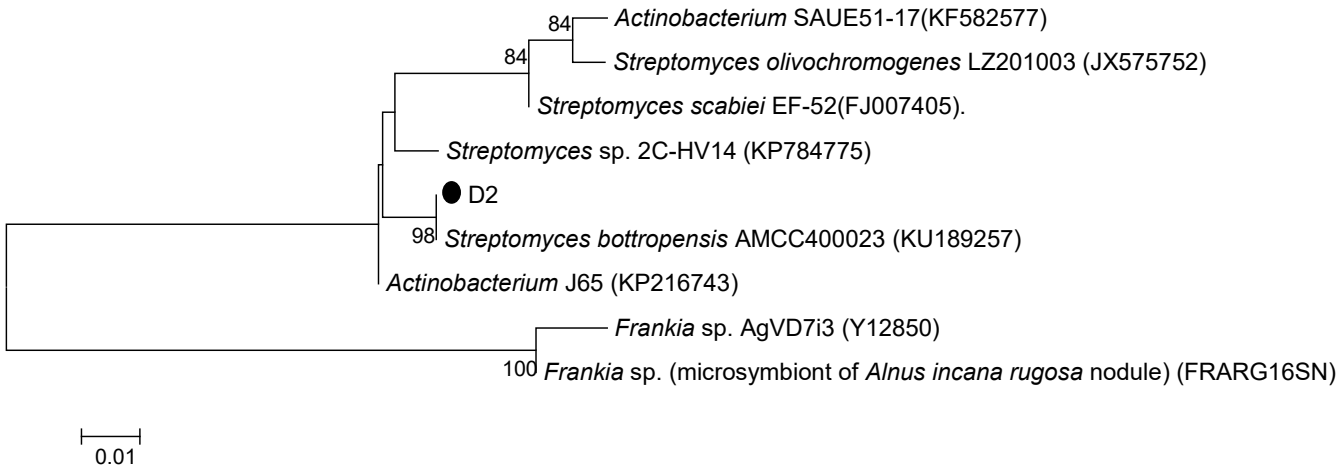

Figure B.1 Phylogenetic tree of 16S rRNA gene sequences of bacterial isolate from *Discaria toumatou* root nodules sampled in New Zealand (●), selected *Actinobacterium*, *Streptomyces* and *Frankia* spp.. This tree was constructed using the MEGA6 software with Maximum likelihood Tamura 3-parameter Gamma distributed with Invariant sites (G+I) method. GenBank accession numbers are in parentheses. Numbers on branches are bootstrap % from 1000 replicates (shown only when ≥ 50%). Scale bar represents 0.01 amino acid substitutions per site (1 substitution per 100 nucleotides).

Streptomyces is the largest genus of Actinobacteria under bacteria kingdom. *Frankia* is another genus which also belongs to Actinobacteria. *Frankia* could associate with some plant species where

they fix nitrogen. Sellstedt and Richau (2013) reported some species of *Streptomyces* have N-fixing capacity as well as *Frankia*, however, *nifH* gene (nitrogen fixation related gene) were not detected in this work. *Streptomyces* was isolated from *Ceanothus velutinus* nodules (Wollum et al., 1966). Allen et al. (1966) reported *Streptomyces* associated with root nodules of *Coriaria* in New Zealand. *Friankia* has been reported in *Discaria toumatou* root (Benson et al., 1996), whereas, there are no reports of *Streptomyces* being isolated from *Discaria toumatou* root nodules. It has been reported that *Streptomyces* can produce antibiotics (de Lima Procópio et al., 2012) which may help plants against disease. *Streptomyces* were not included for more study in this research but it could be interesting for further research that investigates its role in the plant microbiome.

Appendix C

Gene accession number and sequence of isolates in Chapter 3

Table C.1 Gene accession number of the isolates isolated from exotic N-fixers in New Zealand

Gene	ID	Strain	Host	Acc. No.(Genebank)
16S rRNA	SSCV1	Mesorhizobium	*Coronilla varia*	KX770727
16S rRNA	SSCV2	Mesorhizobium	*Coronilla varia*	KX770728
16S rRNA	SSCV3	Mesorhizobium	*Coronilla varia*	KX770729
16S rRNA	SSCV4	Mesorhizobium	*Coronilla varia*	KX770730
16S rRNA	SSAC1	Mesorhizobium	*Astragalus cicer*	KX770731
16S rRNA	SSAC3	Mesorhizobium	*Astragalus cicer*	KX770732
16S rRNA	SSAC2	Ochrobactrum	*Astragalus cicer*	KX770733
16S rRNA	SSAC5	Ochrobactrum	*Astragalus cicer*	KX770734
16S rRNA	SSTag8	Bradyrhizobium	*Cytisus proliferus*	KX770735
16S rRNA	SSTag9	Bradyrhizobium	*Cytisus proliferus*	KX770736
16S rRNA	SSTag10	Bradyrhizobium	*Cytisus proliferus*	KX770737
16S rRNA	SSTag7	Ochrobactrum	*Cytisus proliferus*	KX770738
16S rRNA	SSTag12	Ochrobactrum	*Cytisus proliferus*	KX770739
nifH	SSCV1	Mesorhizobium	*Coronilla varia*	KX774738
nifH	SSCV2	Mesorhizobium	*Coronilla varia*	KX774739
nifH	SSCV3	Mesorhizobium	*Coronilla varia*	KX774740
nifH	SSCV4	Mesorhizobium	*Coronilla varia*	KX774741
nifH	SSAC1	Mesorhizobium	*Astragalus cicer*	KX790340
nifH	SSAC3	Mesorhizobium	*Astragalus cicer*	KX790341
nifH	SSAC2	Ochrobactrum	*Astragalus cicer*	KX790342
nifH	SSAC5	Ochrobactrum	*Astragalus cicer*	KX790343
nifH	SSTag8	Bradyrhizobium	*Cytisus proliferus*	KX790344
nifH	SSTag9	Bradyrhizobium	*Cytisus proliferus*	KX790345
nifH	SSTag10	Bradyrhizobium	*Cytisus proliferus*	KX790346
nifH	SSTag7	Ochrobactrum	*Cytisus proliferus*	KX790347
nifH	SSTag12	Ochrobactrum	*Cytisus proliferus*	KX790348
RecA	SSAC1	Mesorhizobium	*Astragalus cicer*	KX790349
RecA	SSAC3	Mesorhizobium	*Astragalus cicer*	KX790350
RecA	SSAC2	Ochrobactrum	*Astragalus cicer*	KX790351
RecA	SSAC5	Ochrobactrum	*Astragalus cicer*	KX790352
RecA	SSTag8	Bradyrhizobium	*Cytisus proliferus*	KX790353
RecA	SSTag9	Bradyrhizobium	*Cytisus proliferus*	KX790354
RecA	SSTag10	Bradyrhizobium	*Cytisus proliferus*	KX790355
RecA	SSTag7	Ochrobactrum	*Cytisus proliferus*	KX790356
RecA	SSTag12	Ochrobactrum	*Cytisus proliferus*	KX790357

DNA sequences

C1-16S rRNA

AGTCGAGCGCCTCGCAAGAGGAGCGGCAGACGGGTGAGTAACGCGTGGGAATCTACCCATCTCTACGGAACAACTCCGG
GAAACTGGAGCTAATACCGTATACGTCCTTCGGGAGAAAGATTTATCGGAGATGGATGAGCCCGCGTTGGATTAGCTAGT
TGGTGGGGTAATGGCCTACCAAGGCGACGATCCATAGCTGGTCTGAGAGGATGATCAGCCACATTGGGACTGAGACACG
GCCCAAACTCCTACGGGAGGCAGCAGTGGGGAATATTGGACAATGGGCGAAAGCCTGATCCAGCCATGCCGCGTGAGTG
ATGAAGGCCCTAGGGTTGTAAAGCTCTTTCAACGGGGAAGATAATGACGGTAACCGTAGAAGAAGCCCCGGCTAACTTC
GTGCCAGCAGCCGCGGTAATACGAAGGGGGCTAGCGTTGTTCGGAATTACTGGGCGTAAAGCGCACGTAGGCGGATATT
TAAGTCAGGGGTGAAATCCCGGGGCTCAACCCCGGAACTGCCTTTGATACTGGGGATCTCGAGTCCGAGAGAGGTGAGT
GGAATTCCGAGTGTAGAGGTGAAATTCGTAGATATTCGGAGGARCACCAGTGGCGAAGGCGGCTCACTGGCTCGGTACT
GACGCTGAGGTGCGAAAGCGTGGGGAGCAAACAGGATTAGATACCCTGGTAGTCCACGCTGTAAACGATGGAAGCTAG
CCGTTGGCAAGTTTACTTGTCGGTGGCGCAGCTAACGCATTAAGCTTCCCGCCTGGGGAGTACGGTCGCAAGATTAAAAC
TCAAAGGAATTGACGGGGGCCCGCACAAGCGGTGGAGCATGTGGTTTAATTCGAAGCAACGCGCAGAACCTTACCAGCC
CTTGACATCCCGGTCGCGGTTTCCAGAGATGGATTCCTTCAGTTCGGCTGGACCGGTGACAGGTGCTGCATGGCTGTCGT
CAGCTCGTGTCGTGAGATGTTGGGTTAAGTCCCGCAACGAGCGCAACCCTCGCCCTTAGTTGCCAGCATTAAGTTGGGCA
CTCTAAGGGGACTGCCGGTGATAAGCCGAGAGGAAGGTGGGGATGACGTCAAGTCCTCATGGCCCTTACGGGCTGGGCT
ACACACGTGCTACAATGGTGGTGACAGTGGGCAGCGAGACCGCGAGGTCGAGCTAATCTCCAAAAGCCATCTCAGTTCG
GATTGCACTCTGCAACTCGAGTGCATGAAGTTGGAATCGCTAGTAATCGCGGATCAGCATGCCGCGGTGAATACGTTCCC
GGGCCTTGTACACACCGCCCGTCACACCATGGGAGTTGGTTTTACCCGAAGGCGCTGTGCTAACCGCAAGGAGGCAGGC
GACCACA

C2-16S rRNA

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CCGTATACGTCCTTCGGGAGAAAGATTTATCGGAGATGGATGAGCCCGCGTTGGATTAGCTAGTTGGTGGGGTAATGGC
CTACCAAGGCGACGATCCATAGCTGGTCTGAGAGGATGATCAGCCACATTGGGACTGAGACACGGCCCAAACTCCTACG
GGAGGCAGCAGTGGGGAATATTGGACAATGGGCGAAAGCCTGATCCAGCCATGCCGCGTGAGTGATGAAGGCCCTAGG
GTTGTAAAGCTCTTTCAACGGGGAAGATAATGACGGTAACCGTAGAAGAAGCCCCGGCTAACTTCGTGCCAGCAGCCGC
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TGGTGGTGACAGTGGGCAGCGAGACCGCGAGGTCGAGCTAATCTCCAAAAACCATCTCAGTTCGGATTGCACTCTGCAAC
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C3-16S rRNA

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TCGAGTGCATGAAGTTGGAATCGCTAGTAATCGCGGATCAGCATGCCGCGGTGAATACGTTCCCGGGCCTTGTACACACC
GCCCGTCACACCATGGGAGTTGGTTTTACCCGAAGGCGCTGTGCTAACCGCAAGGAGGCA

C4-16S rRNA

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ACC

A1-16S rRNA

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A2-16S rRNA

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GTGAGTGGAATTCCGAGTGTAGAGGTGAAATTCGTAGATGTTCGGAGGAACACCAGTGGCGAAGGCGGCTCACTGGACC
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A3-16S rRNA

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AAGGCGACGATCCATAGCTGGTCTGAGAGGATGATCAGCCACATTGGGACTGAGACACGGCCCAAACTCCTACGGGAGG
CAGCAGTGGGGAATATTGGACAATGGGCGAAAGCCTGATCCAGCCATGCCGCGTGAGTGATGAAGGCCCTAGGGTTGTA
AAGCTCTTTCAACGGTGAAGATAATGACGGTAACCGTAGAAGAAGCCCCGGCTAACTTCGTGCCAGCAGCCGCGGTAATA
CGAAGGGGGCTAGCGTTGTTCGGAATTACTGGGCGTAAAGCGCACGTAGGCGGATATTTAAGTCAGGGGTGAAATCCCG
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AAATTCGTAGATATTCGGAGGAACACCAGTGGCGAAGGCGGCTCACTGGCTCGGTACTGACGCTGAGGTGCGAAAGCGT
GGGGAGCAAACAGGATTAGATACCCTGGTAGTCCACGCTGTAAA

A5-16S rRNA

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GTTGGTGGGGTAAAGGCTCACCAAGGCGACGATCCATAGCTGGTCTGAGAGGATGATCAGCCACACTGGGACTGAGACA
CGGCCCAGACTCCTACGGGAGGCAGCAGTGGGGAATATTGGACAATGGGCGCAAGCCTGATCCAGCCATGCCGCGTGA
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GTGGAATTCCGAGTGTAGAGGTGAAATTCGTAGATATTCGGAGGAACACCAGTGGCGAAGGCGGCTCACTGGCTCATTA
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T7-16S rRNA

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T8-16S rRNA

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T9-16S rRNA

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CAACCCCCGTCCTTAGTTGCTACCATTTAGTTGAGCACTCTAAGGAGACTGCCGGTGATAAGCCGCGAGGAAGGTGGGGA
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AATCATGGATCAGCACGCCACGGTGAATACGTTCCCGGGCCTTGTACACACCGCCCGTCACACCATGGGAGTTGGTTTTA
CCTGAAGACGGTGCGCTAACCAGCAATGGAGGCAGCCG

T10-16S rRNA

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TGCCTTTGATACTGAAGATCTTGAGTTCGGGAGAGGGTGAGTGGAACTGCGAGTGTAGAGGTGAAATTCGTAGATATTC
GCAAGAACACCAGTGGCGAAGGCGGCTCACTGGCCCGATACTGACGCTGAGGCACGAAAGCGTGGGGAGCAAACAGGA
TTAGATACCCTGGTAGTCCACGCCGTAAACGATGAATGCCAGCCGTTAGTGGGTTTACTCACTAGTGGCGCAGCTAACGC
TTTAAGCATTCCGCCTGGGGAGTACGGTCGCAAGATTAAAACTCAAAGGAATTGACGGGGGCCCGCACAAGCGGTGGAG
CATGTGGTTTAATTCGGCGCTACGCGCAGAACCTTACCAGCCCTTGGACATGTCCAGGAACGGTCGCAGAGATGTGACCT
TCTCTTCGGAGCCTGGAACACAGGTGCTGCATGGCTGTCGTCAGCTCGTGTCGTGAGATGTTGGGTTAAGTCCCGCAACG
AGCGCAACCCCCGTCCTTAGTTGCTACCATTTAGTTGAGCACTCTAAGGAGACTGCCGGTGATAAGCCGCGAGGAAGGTG
GGGATGACGTCAAGTCCTCATGGCCCTTACGGGCTGGGCTACACACGTGCTACAATGGCGGTGACAATGGGATGCTAAG
GGGCGACCCTTCGCAAATCTCAAAAAGCCGTCTCAGTTCGGATTGGGCTCTGCAACTCGAGCCCATGAAGTTGGAATAGA
GTTTAATCGTGGATCAGCACGCCACGGTGAATACGTTCCCGGGCCTTGTACACACCGCCCGTCACACCATGGGAGTTGGT
TTTACCTGAAGACGGTGCGCTAACCAGCA

T12-16S rRNA

TTTTGCTACGGAACAACAGTTGGAAACGACTGCTAATACCGTATGTGCCCTTCGGGGGAAAGATTTATCGGCAAAGGATG
AGCCCGCGTTGGATTAGCTAGTTGGTGAGGTAAAGGCTCACCAAGGCGACGATCCATAGCTGGTCTGAGAGGATGATCA
GCCACACTGGGACTGAGACACGGCCCAGACTCCTACGGGAGGCAGCAGTGGGGAATATTGCACAATGGGCGCAAGCCT
GATCCAGCCATGCC

C1-nifH

AGTGCTGCATCTGGCGGCTCAGGAGGGTTCGGTGGAGGATCTCGAACTCCAGGACGTGCTCAAGATCGGCTACAGAGGC
ATCAAGTGCGTGGAGTCCGGCGGTCCCGAGCCGGGTGTCGGCTGCGCCGGCCGCGGCGTCATCACCTCGATTAACTTTCT
TGAGGAGAACGGCGCTTACGATGATGTCGACTATGTATCCTACGATGTGCTCGGCGATGTTGTGTGCGGCGGCTTCGCAA
TGCCGATCCGCGAGGGCAAGGCTCAGGAAATCTATATCGTGATGTCC

C2-nifH

GCGCAGGATACAGTGCTGCATCTGGCGGCTCAGGAGGGTTCGGTGGAGGATCTCGAACTCCAGGACGTGCTCAAGATCG
GCTACAGAGGCATCAAGTGCGTGGAGTCCGGCGGTCCCGAGCCGGGTGTCGGCTGCGCCGGGCGCGGGGTCATCACCT
CGATTAACTTTCTTGAGGAGAACGGCGCTTACGATGATGTCGACTATGTATCCTACGATGTGCTCGGCGATGTTGTGTGCG
GCGGCTTCGCAATGCCGATCCGCGAGGGCAAGGCTCAGGAAATCTATATCGTGATGTCCGGTGAGATGATGGCGAT

C3-nifH

AGGATACAGTGCTGCATCTGGCGGCTCAGGAGGGTTCGGTGGAGGATCTCGAACTCCAGGACGTGCTCAAGATCGGCTA
CAGAGGCATCAAGTGCGTGGAGTCCGGCGGTCCCGAGCCGGGTGTCGGCTGCGCCGGGCGCGGCGTCATCACCTCGATT
AACTTTCTTGAGGAGAACGGCGCTTACGATGATGTCGACTATGTATCCTACGATGTGCTCGGCGATGTTGTGTGCGGCGG
CTTCGCGATGCCGATCCGCGAGGGCAAGGCTCAGGAAATCTATATCGTGATGTCCG

C4-nifH

AGGATACAGTGCTGCATCTGGCGGCTCAGGAGGGTTCGGTGGAGGATCTCGAACTCCAGGACGTGCTCAAGATCGGCTA
CAGAGGCATCAAGTGCGTGGAGTCCGGCGGTCCCGAGCCGGGTGTCGGCTGCGCCGGCCGCGGCGTCATCACCTCGATT
AACTTTCTTGAGGAGAACGGCGCTTACGATGATGTCGACTATGTATCCTACGATGTGCTCGGCGATGTTGTGTGCGGCGG
CTTCGCAATGCCGATCCGCGAGGGCAAGGCTCAGGAAATCTATATCGTGATGTCCGGCGAGATGATGGC

A1-nifH

ACAGTGCTGCATCTGGCGGCTCAGGAGGGTTCGGTGGAGGATCTCGAACTCCAGGACGTGCTCAAGATCGGCTACAGAG
GCATCAAGTGCGTGGAGTCCGGCGGTCCCGAGCCGGGTGTCGGCTGCGCCGGCCGCGGCGTCATCACCTCGATTAACTTT
CTTGAGGAGAACGGCGCTTACGATGATGTCGACTATGTATCCTACGATGTGCTCGGCGATGTTGTGTGCGGCGGCTTCGC
AATGCCGATCCGCGAGGGCAAGGCTCAGGAAATCTATATCGTGATGTCCG

A2-nifH

AGTGCTGCATCTGGCGGCTCAGGAGGGTTCGGTGGAGGATCTCGAACTCCAGGACGTGCTCAAGATCGGCTACAGAGGC
ATCAAGTGCGTGGAGTCCGGCGGTCCCGAGCCGGGTGTCGGCTGCGCCGGCCGCGGCGTCATCACCTCGATTAACTTTCT
TGAGGAGAACGGCGCTTACGATGATGTCGACTATGTATCCTACGATGTGCTCGGCGATGTTGTGTGCGGCGGCTTCGCAA
TGCCGATCCGCGAGGGCAAGGCTCAGGAAATCTATATCGTGATGTCC

A3-nifH

CAGGATACAGTGCTGCATCTGGCGGCTCAGGAGGGTTCGGTGGAGGATCTCGAACTCCAGGACGTGCTCAAGATCGGCT
ACAGAGGCATCAAGTGCGTGGAGTCCGGCGGTCCCGAGCCGGGTGTCGGCTGCGCCGGCCGCGGCGTCATCACCTCGAT
TAACTTTCTTGAGGAGAACGGCGCTTACGATGATGTCGACTATGTATCCTACGATGTGCTCGGCGATGTTGTGTGCGGCG
GCTTCGCAATGCCGATCCGCGAGGGCAAGGCTCAGGAAATCTATATCGTGATGTCCG

A5-nifH

ATACAGTGCTGCATCTGGCGGCTCAGGAGGGTTCGGTGGAGGATCTCGAACTCCAGGACGTGCTCAAGATCGGCTACAG
AGGCATCAAGTGCGTGGAGTCCGGCGGTCCCGAGCCGGGTGTCGGCTGCGCCGGCCGCGGCGTCATCACCTCGATTAAC
TTTCTTGAGGAGAACGGCGCTTACGATGATGTCGACTATGTATCCTACGATGTGCTCGGCGATGTTGTGTGCGGCGGCTT
CGCAATGCCGATCCGCGAGGGCAAGGCTCAGGAAATCTATATCGTGATGTCCG

T7-nifH

TCGAGGACCTCGAAATCGAGGACGTCATCAAGCTCGGCTACAAGGACATTCGATGCGTCGAGTCCGGCGGTCCGGAGCC
GGGGGTCGGGTGCGCCGGAAGAGGCGTGATCACTTCCATAAACTTTCTGGAGGAGAATGGCGCCTATGAGGACATCGAC
TACGTCTCTTACGACGTGCTCGGCGACGTCGTCTGCGGCGGCTTCGCGATGCCTATCCGCGAGAACAAGGCACAGGAAAT
CTACATCGTGATGTCCG

T8-nifH

ATTCTGAGCCTGGCGGCGAATGCCGGCAGCGTCGAGGACCTCGAAATCGAGGACGTCATCAAGCTCGGCTACAAGGACA
TTCGATGCGTCGAGTCCGGCGGTCCGGAGCCAGGGGTCGGGTGCGCCGGAAGAGGCGTGATCACTTCCATTAACTTTCT
GGAGGAGAATGGCGCCTATGAGGGCATCGACTACGTCTCTTACGACGTGCTCGGCGACGTCGTCTGCGGCGGCTTCGCG
ATGCCTATCCGCGAGAACAAGGCGCAGGAAATCTACATCGTGATGTCCG

T9-nifH

ATTCTGAGCCTGGCGGCGAGTGCCGGCAGCGTCGAGGACCTCGAAATCGAGGACGTCATCAAGCTCGGCTACAAGGACA
TTCGATGCGTCGAGTCCGGCGGTCCGGAGCCAGGGGTCGGGTGCGCCGGAAGAGGCGTGATCACTTCCATTAACTTTCT
GGAGGAGAACGGCGCCTACGAGGGCATCGACTACGTCTCTTACGACGTGCTCGGCGACGTCGTCTGCGGCGGCTTCGCG
ATGCCTATCCGCGAGAACAAGGCGCAGGAAATCTACATCGTGATGTCC

T10-nifH

CCATTCTGAGCCTGGCGGCGAGTGCCGGCAGCGTCGAGGACCTCGAAATCGAGGACGTCATCAAGCTCGGCTACAAGGA
CATTCGATGCGTCGAGTCCGGCGGTCCGGAGCCAGGGGTCGGGTGCGCCGGAAGAGGCGTGATCACTTCCATTAACTTT
CTGGAGGAGAACGGCGCCTACGAGGGCATCGACTACGTCTCTTACGACGTGCTCGGCGACGTCGTCTGCGGCGGCTTCG
CGATGCCTATCCGCGAGAACAAGGCGCAGGAAATCTACATCGTGATGTCC

T12-nifH

CGAGGACCTCGAAATCGAGGACGTCATCAAGCTCGGCTACAAGGACATTCGATGCGTCGAGTCCGGCGGTCCGGAGCCA
GGGGTCGGGTGCGCCGGAAGAGGCGTGATCACTTCCATTAACTTTCTGGAGGAGAATGGCGCCTATGAGGGCATCGACT
ACGTCTCTTACGACGTGCTCGGCGACGTCGTCTGCGGCGGCTTCGCGATGCCTATCCGCGAGAACAAGGCGCAGGAAATC
TACATCGTGATGTCCGG

A1-recA

GTCGACGCCGAGCATGCGCTCGACCCGGTCTATGCCCGCAAGCTCGGCGTCGACCTCGAAAACCTGCTGATCTCGCAGCC
CGACACCGGCGAGCAGGCGCTGGAGATCTGCGACACGCTGGTGCGCTCCGGCGCCATCGACGTGCTGGTGGTCGATTCG
GTTGCGGCACTGACGCCGCGCGCCGAAATCGAAGGCGAGATGGGCGATTCGCTGCCCGGCCTGCAGGCGCGTCTGATGA
GCCAGGCGCTGCGCAAGCTGACCGCCTCGATCTCGCGCTCC

A2-recA

TCGCCTTGGTCAGAATGATCAGGTAGTAGAGATTGAAACGGTGTCGACCGGTTCGCTTTCTCTCGATATTGCATTGGGCGT
CGGCGGTCTGCCCAAGGGACGTATCGTAGAAATTTATGGTCCGGAAAGCTCGGGTAAGACGACGCTTGCACTCCACACG
ATTGCAGAAGCTCAGAAAAAGGGCGGTATCTGCGCATTTGTGGATGCGGAACATGCTCTTGATCCGGTCTATGCCCGTAA
GCTTGGGGTCGATCTTGAGAACCTGCTGATCTCGCAGCCAGATACTGGTGAGCAGGCGCTTGAAATCACGGATACGCTTG
TGCGGTCTGGCGCAATTGATGTTCTGGTTATCGACTCGGTCGCAGCTCTGACGCCACGCGCCGAAATCGAAGGTGAAATG
GGCGATTCCTTGCCTGGTCTTCAGGCGCGATTGATGAGCCAGGCATTGCGCAAGCTCACGGCTTCGATCTCGCGTTCGAA
CTG

A3-recA

CCGAGCATGCGCTCGACCCGGTCTATGCCCGCAAGCTCGGCGTCGACCTCGAAAACCTGCTGATCTCGCAGCCCGACACC
GGCGAGCAGGCGCTGGAGATCTGCGACACGCTGGTGCGCTCCGGCGCCATCGACGTGCTGGTGGTCGATTCGGTTGCGG
CACTGACGCCGCGCGCCGAAATCGAAGGCGAGATGGGCGATTCGCTGCCCGGCCTGCAGGCGCGTCTGATGAGCCAGG
CGCTGCGCAAGCTGACCGCCTCGATCTCGCGCT

A5-recA

CCTTGGTCAGAATGATCAGGTAGTAGAGATTGAAACGGTGTCGACCGGTTCGCTTTCTCTCGATATTGCATTGGGCGTCG
GCGGTCTGCCCAAGGGACGTATCGTAGAAATTTATGGTCCGGAAAGCTCGGGTAAGACGACGCTTGCACTCCACACGATT
GCAGAAGCTCAGAAAAAGGGCGGTATCTGCGCATTTGTGGATGCGGAACATGCTCTTGATCCGGTCTATGCCCGTAAGCT
TGGGGTCGATCTTGAGAACCTGCTGATCTCGCAGCCAGATACTGGTGAGCAGGCGCTTGAAATCACGGATACGCTTGTGC
GGTCTGGCGCAATTGATGTTCTGGTTATCGACTCGGTCGCAGCTCTGACGCCACGCGCCGAAATCGAAGGTGAAATGGGC
GATTCCTTGCCTGGTCTTCAGGCGCGATTGATGAGCCA

T7-recA

TCAGAATGATCAGGTAGTAGAGATTGAAACGGTGTCGACCGGTTCGCTTTCTCTCGATATTGCATTGGGCGTCGGCGGTC
TGCCCAAGGGACGTATCGTGGAAATTTACGGTCCGGAAAGCTCGGGTAAGACGACGCTTGCACTCCACACGATTGCAGA
AGCTCAGAAAAAGGGCGGAATCTGCGCATTAGTGGATGCGGAACATGCTCTTGACCCGGTCTATGCCCGTAAGCTTGGG
GTCGATCTTGAGAACCTGCTGATCTCGCAGCCAGATACTGGTGAGCAGGCGCTTGAAATC

T8-recA

CGCTGCACACGGTGGCGGAAGCGCAGAAGAAGGGCGGAATCTGCGCCTTCATCGACGCCGAGCACGCGCTCGACCCGG
TCTATGCGCGCAAGCTGGGCGTCAACATCGACGAGCTCCTGATTTCGCAGCCGGACACGGGCGAGCAGGCGCTGGAAAT
CTGCGACACGCTGGTGCGCTCGGGGGCGGTGGACGTGCTGGTGGTCGATTCGGTCGCGGCTCTGGTGCCGAAGGCCGA
GCTCGAAGGCGAGATGGGCGATGCGCTGCCGGGTCTCCAGGCCCGTCTAATGAGCCAGGCGCTGCGCAAGCTGACGGC
CTCCATCAAC

T9-recA

CGCTGCACACGGTGGCGGAAGCGCAGAAGAAGGGCGGAATCTGCGCCTTCATTGACGCCGAGCACGCGCTCGACCCGGT
CTATGCGCGCAAGCTGGGCGTCAACATCGACGAGCTCCTGATTTCGCAGCCGGACACGGGCGAGCAGGCGCTGGAAATC
TGCGACACGCTGGTGCGCTCGGGTGCGGTAGACGTGCTGGTGGTCGATTCGGTCGCGGCTCTGGTGCCGAAGGCCGAGC
TCGAGGGCGAGATGGGCGACGCGCTGCCGGGTCTCCAGGCCCGTCTGATGAGCCAGGCGCTGCGCAAGCTGACGGCCT
CCATCAAC

T10-recA

CGCTGCACACGGTGGCGGAAGCGCAGAAGAAGGGCGGAATCTGCGCCTTCATCGACGCCGAGCACGCGCTCGACCCGG
TCTATGCGCGCAAGCTGGGCGTCAACATCGACGAGCTCCTGATTTCGCAGCCGGACACGGGCGAGCAGGCGCTGGAAAT
CTGCGACACGCTGGTGCGCTCGGGTGCGGTAGACGTGCTGGTGGTCGATTCGGTCGCGGCTCTGGTGCCGAAGGCCGAG
CTCGAGGGCGAGATGGGCGACGCGCTGCCGGGTCTCCAGGCCCGTCTGATGAGCCAGGCGCTGCGCAAGCTGACGGCC
TCCATCAAC

T12-recA

GGTCAGAATGATCAGGTAGTAGAGATTGAAACGGTGTCGACCGGTTCGCTTTCTCTCGATATTGCATTGGGCGTCGGCGG
TCTGCCCAAGGGACGTATCGTAGAAATTTATGGTCCGGAAAGCTCGGGTAAGACGACGCTTGCACTCCACACGATTGCAG
AAGCTCAGAAAAAGGGCGGTATCTGCGCATTTGTGGATGCGGAACATGCTCTTGATCCGGTCTATGCCCGTAAGCTTGGG
GTCGATCTTGAGAACCTGCTGATCTCGCAGCCAGATACTGGTGAGCAGGCGCTTGAAATCACGGATACGCTTGTGCGGTC
TGGCGCAATTGATGTTCTGGTTATCGACTCGGTCGCAGCTCTGACGCCACGCGCCGAAATCGAAGGTGAAATGGGCGATT
CCTTGCCTGGTCTTCAGGCGCGATTGATGAGCCAGGCATTGCGCAAGCTCAC

Appendix D

Plant species, soil chemistry and other details in Eyrewell reserve

Table D.1 Indigenous species of Eyrewell Reserve (Ecroyd & Brockerhoff, 2005).

Species	1972	1995	2001-2003	Shrubland	Pasture	Pine forest	Species	1972	1995	2001-2003	Shrubland	Pasture	Pine forest
Acaena novae-zealandiae	†	x					*Leptinella pusilla* [3]	x	x	x	x		x
Aciphylla subflabellata [1, 2]	x						*Leptinella serrulata* [2, 3]	x					
Asplenium flabellifolium			x				*Leptospermum scoparium*	x	x				
Brachyglottis bellidioides [3]	x	x	x				*Leucopogon fraseri* [3]	x	x	x			x
Caladenia lyallii	x						*Luzula rufa* var. *rufa*	x		x			
Carex breviculmis	x	x	x			x	*Melicytus alpinus* [3]	†		x			
Carex colensoi	x						*Mentha cunninghamii*	x					
Carex goyenii			x			x	*Microlaena stipoides*	x	x	x	x		x
Carmichaelia australis [3]	x	x	x	x		x	*Microsorum pustulatum*			x			
Celmisia gracilenta	x	x	x			x	*Microtis unifolia*	x		x			x
Clematis quadribracteolata			x			x	*Muehlenbeckia axillaris*	x	x				x
Coprosma crassifolia			x				*Nertera setulosa*	x	x	x			
Coprosma intertexta [2]	†		x				*Ophioglossum coriaceum*	x					
Coprosma propinqua	x	x	x				*Oxalis exilis* [3]	x	x	x	x		
Coprosma rhamnoides	x	x					*Ozothamnus leptophyllus* [3]	†	x				
Cyathodes juniperina	x	x	x			x	*Pimelea* sp. (seedling)	x					
Deyeuxia avenoides	x	x	x			x	*Poa cita* [3]	x	x	x			
Dichelachne crinita	x	x	x	x		x	*Poa pusilla*			x			
Dichondra brevifolia	x					x	*Pomaderris* aff. *phylicifolia* [3, 6]	x		x			x
Dichondra repens	x	x	x	x		x	*Prasophyllum colensoi*	x					
Discaria toumatou	†	x	x				*Pteridium esculentum*						x
Epilobium alsinoides ssp. *atriplicifolium*			x				*Pterostylis tristis* [2, 3]	x					
Epilobium cinereum			x				*Pyrrhanthera exigua*	x					
Euchiton audax [3]	x	x	x	x		x	*Ranunculus multiscapus* [3]	x					
Euchiton collinus [3]						x	*Raoulia monroi*	x					
Euchiton sphaericus [1]	x						*Rytidosperma clavatum* [3]	x			x		x
Festuca novae-zelandiae	x		x				*Rytidosperma gracile* [3]	x		x			x
Galium perpusillum	x						*Rytidosperma unarede* [3]	x	x	x	x		x
Galium propinquum	x	x	x				*Scleranthus uniflorus* [1]	x					
Geranium microphyllum	x		x	x			*Senecio glomeratus*			x	x		x
Geranium sessiliflorum	x						*Senecio minimus*			x			
Gonocarpus incanus [4]	x						*Senecio quadridentatus*						x
Gonocarpus micranthus [3]	x						*Solanum laciniatum*			x			
Helichrysum filicaule	x						*Stackhousia minima*	x	x	x			
Hydrocotyle moschata [5]	x	x	x				*Thelymitra longifolia*	x		x			x
Hypericum gramineum	x	x	x			x	*Thelymitra pauciflora*	x	x[7]	x			
Hypolepis ambigua			x				*Viola cunninghamii*	x					
Kunzea ericoides [3]	x	x	x	x		x	*Wahlenbergia albomarginata*	x	x				
Lagenifera strangulata [3]	x	x	x				*Wahlenbergia violacea* [3]	x	x	x	x		x

† Species growing in the area but not recorded from the reserve.

[1] Although not reported by Molloy *et al.* 1972 as growing in the reserve the locality given on the voucher in the Allan Herbarium (CHR) is "Eyrewell Reserve".

[2] Threatened species, see de Lange *et al.* (2004).

[3] Species recorded under another name by Molloy and Ives (1972) or Meurk *et al.* (1995)

[4] This species is on the voucher sheet (CHR 386268) collected by B.P.J. Molloy together with *Gonocarpus micranthus*.

[5] Meurk *et al.* (1995) record *Hydrocotyle novae-zeelandiae* in Eyrewell Reserve but we found *H. moschata* and suggest that these two species have been confused.

[6] *Pomaderris* aff. *phylicifolia* is the very common species which reaches its southern limit at Eyrewell (Brandon *et al.* 2004, Peter de Lange pers. comm.) and is not the species recorded as threatened by de Lange *et al.* (2004).

[7] Identification uncertain.

Table D.2 Inroduced species of Eyrewell Reserve (Ecroyd & Brockerhoff, 2005).

Species	1972	1995	2001-2003	Shrubland	Pasture	Pine forest
Acaena agnipila						x
Acer pseudoplatanus (1 seedling)			x			
Achillea millefolium			x	x	x	x
Agrostis capillaris[1]	x	x	x	x	x	x
Agrostis stolonifera					x	x
Aira caryophyllea	x	x	x	x		x
Anthoxanthum odoratum	x	x	x	x	x	x
Anthriscus caucalis			x			
Aphanes inexspectata			x			
Bromus stamineus			x			
Buddleja davidii			x			
Cardamine hirsuta			x			
Cerastium fontanum ssp. *vulgare*[1]	x	x	x	x		x
Cerastium glomeratum			x			x
Chenopodium album			x			
Cirsium arvense			x			x
Cirsium vulgare	x	x	x	x		x
Conyza sumatrensis[1]			x			
Cotoneaster simonsii			x			
Crepis capillaris	x	x	x	x	x	x
Crepis vesicaria ssp. *taraxifolia*			x			
Cynosurus echinatus			x			
Cytisus scoparius	x	x	x			x
Dactylis glomerata			x	x		
Digitalis purpurea			x			
Dryopteris filix-mas			x			
Duchesnea indica			x			
Echium vulgare					x	
Elymus rectisetus[1]	x	x	x	x	x	x
Epilobium ciliatum			x	x		
Fallopia convolvulus			x			
Festuca rubra			x			
Fragaria vesca			x			
Galium aparine			x			
Hieracium aurantiacum						x
Hieracium lepidulum[1]	x		x			x
Hieracium pilosella	x	x	x	x		x
Hieracium praealtum	x	x	x			x
Holcus lanatus	x		x	x	x	
Hypochoeris radicata	x	x	x	x	x	x
Leucanthemum vulgare						x
Leontodon taraxacoides	x		x			

Species	1972	1995	2001-2003	Shrubland	Pasture	Pine forest
Linum catharticum	x					
Lolium perenne			x	x	x	
Luzula flaccida[2]			x			
Mycelis muralis			x			
Pelargonium inodorum	x		x	x		
Pinus radiata			x			x
Plantago lanceolata			x	x	x	x
Poa pratensis			x		x	
Prunus cerasus[2]			x			
Prunus sp. (seedlings)			x			
Ribes sanguineum			x			
Rosa rubiginosa	x		x			x
Rubus fruticosus agg.			x			
Rubus idaeus			x			
Rubus laciniatus						x
Rubus sp.			x			
Rumex acetosella	x	x	x	x	x	x
Sagina apetala	x					
Sambucus nigra			x			
Senecio jacobaea			x			
Sisyrinchium striatum			x			
Solanum dulcamara			x			
Sonchus asper			x	x		
Sonchus oleraceus			x			
Spergularia rubra	x					
Stellaria media			x			
Tanacetum parthenium			x			
Taraxacum officinale			x	x		x
Trifolium arvense	x					
Trifolium dubium	x	x	x			
Trifolium glomeratum	x					
Trifolium repens	x	x	x	x	x	x
Trifolium striatum	x					
Trifolium subterraneum	x	x			x	x
Ulex europaeus	x	x	x	x		x
Urtica urens			x			
Verbascum thapsus	x	x	x	x		
Veronica arvensis			x			
Vicia sativa[1]	x		x			x
Vicia tetrasperma			x			
Viola odorata			x			
Vulpia bromoides	x		x	x		

[1] Species recorded under another name by Molloy and Ives (1972) or Meurk *et al.* (1995).

[2] Identification uncertain.

Table D.3 Soil chemistry in Eyrewell area (Ngai Tahu Ltd).

Ballance agri-nutrients

Hill Laboratories
BETTER TESTING BETTER RESULTS

R J Hill Laboratories Limited
1 Clyde Street
Private Bag 3205
Hamilton 3240, New Zealand

Tel +64 7 858 2000
Fax +64 7 858 2001
Email mail@hill-labs.co.nz
Web www.hill-labs.co.nz

ANALYSIS REPORT

Client:	Ngai Tahu Forest Estates Ltd	Lab No:	1140193 s2chpv1
Address:	PO Box 130060 CHRISTCHURCH 8141	Date Registered:	29-May-2013
		Date Reported:	05-Jun-2013
		Quote No:	54854
		Order No:	P. Roberts
		Client Reference:	4036010
		Add. Client Ref:	Eyrewell
		Submitted By:	Mr R Williams (KA)

Sample Name: Sth Harrington Rd West
Lab Number: 1140193.1
Sample Type: SOIL Mixed Pasture, Dry Stock (Sed.) (S186)

Analysis		Level	Optimum	Below Optimum	Above
pH	pH Units	5.2	5.8 - 6.0		
Olsen Phosphorus	mg/L	12	20 - 30		
Potassium	MAF units	8	6 - 8		
Calcium	MAF units	2	4 - 6		
Magnesium	MAF units	19	8 - 10		
Sodium	MAF units	3			
Sulphate Sulphur	mg/kg	7	10 - 12		
Aluminium ($CaCl_2$ Extractable)	mg/kg	13.7	0.0 - 3.0		
Total Carbon	%	4.0			
Total Nitrogen	%	0.19	0.30 - 0.60		
C/N Ratio*		20.6			
Soil Sample Depth*	mm	0-150			
Base Saturation	%	K 2.8	Ca 11	Mg 6.1	Na 0.5
	me/100g	K 0.49	Ca 2.0	Mg 1.08	Na 0.09
Additional Properties		Cation Exchange Capacity (me/100g)			18
		Total Base Saturation (%)			20
		Volume Weight (g/mL)			0.78

Sample Name: Davis Rd East (Blue 8B)
Lab Number: 1140193.2
Sample Type: SOIL Mixed Pasture, Dry Stock (Sed.) (S186)

Analysis		Level	Optimum	Below Optimum	Above
pH	pH Units	5.3	5.8 - 6.0		
Olsen Phosphorus	mg/L	9	20 - 30		
Potassium	MAF units	8	6 - 8		
Calcium	MAF units	2	4 - 6		
Magnesium	MAF units	19	8 - 10		
Sodium	MAF units	4			
Sulphate Sulphur	mg/kg	5	10 - 12		
Aluminium ($CaCl_2$ Extractable)	mg/kg	11.5	0.0 - 3.0		
Total Carbon	%	3.8			
Total Nitrogen	%	0.19	0.30 - 0.60		
C/N Ratio*		19.4			
Soil Sample Depth*	mm	0-150			
Base Saturation	%	K 2.7	Ca 12	Mg 5.9	Na 0.6
	me/100g	K 0.43	Ca 2.0	Mg 0.95	Na 0.09
Additional Properties		Cation Exchange Capacity (me/100g)			16
		Total Base Saturation (%)			22
		Volume Weight (g/mL)			0.87

Sample Name: Sth Hunter Rd East (Green 15)
Lab Number: 1140193.3
Sample Type: SOIL Mixed Pasture, Dry Stock (Sed.) (S186)

Analysis		Level	Optimum	Below Optimum	Above
pH	pH Units	5.3	5.8 - 6.0		
Olsen Phosphorus	mg/L	11	20 - 30		
Potassium	MAF units	7	6 - 8		
Calcium	MAF units	1	4 - 6		
Magnesium	MAF units	15	8 - 10		
Sodium	MAF units	3			
Sulphate Sulphur	mg/kg	6	10 - 12		
Aluminium ($CaCl_2$ Extractable)	mg/kg	12.9	0.0 - 3.0		
Total Carbon	%	3.5			
Total Nitrogen	%	0.16	0.30 - 0.60		
C/N Ratio*		21.1			
Soil Sample Depth*	mm	0-150			
Base Saturation	%	K 2.7	Ca 10	Mg 5.5	Na 0.6
	me/100g	K 0.43	Ca 1.5	Mg 0.87	Na 0.09
Additional Properties		Cation Exchange Capacity (me/100g)			16
		Total Base Saturation (%)			19
		Volume Weight (g/mL)			0.77

Sample Name: Sth Hunter Rd West (Green 9B)
Lab Number: 1140193.4
Sample Type: SOIL Mixed Pasture, Dry Stock (Sed.) (S186)

Analysis		Level	Optimum	Below Optimum	Above
pH	pH Units	5.2	5.8 - 6.0		
Olsen Phosphorus	mg/L	12	20 - 30		
Potassium	MAF units	7	6 - 8		
Calcium	MAF units	1	4 - 6		
Magnesium	MAF units	15	8 - 10		
Sodium	MAF units	3			
Sulphate Sulphur	mg/kg	7	10 - 12		
Aluminium ($CaCl_2$ Extractable)	mg/kg	16.3	0.0 - 3.0		
Total Carbon	%	4.2			
Total Nitrogen	%	0.20	0.30 - 0.60		
C/N Ratio*		20.9			
Soil Sample Depth*	mm	0-150			
Base Saturation	%	K 2.5	Ca 8	Mg 5.0	Na 0.6
	me/100g	K 0.40	Ca 1.2	Mg 0.79	Na 0.09
Additional Properties		Cation Exchange Capacity (me/100g)			16
		Total Base Saturation (%)			16
		Volume Weight (g/mL)			0.82

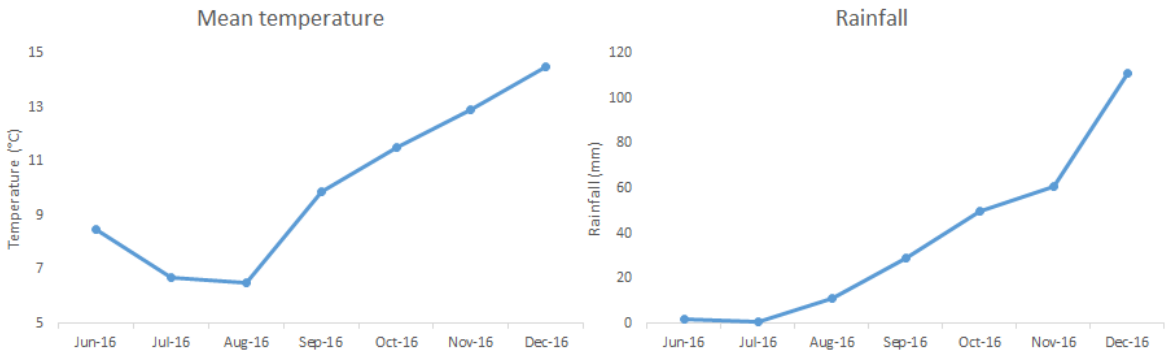

Figure D.4 Air temperature and precipitation of Eyrewell area for the field experiment (Chapter 6) from planting (June, 2016) to harvesting (December, 2016). Data were collected from NIWA, New Zealand.

Appendix E

N-fixers and their surrounding species

Table E.1 List of N-fixers (shown in boldface) and surrounding species at different locations (L1-L7) in Canterbury.

Location	Species	Location	Species
L1 Mt Cass	***Carmichaelia australis*** ***Discaria toumatou*** *Coprosma propinqua* *Cytisus scoparius* (exotic) *Asplenium flabellifolium* *Wahlenbergia albomarginata* *Muehlenbeckia complexa* *Poa cita* *Muehlenbeckia axillaris*	L5 Port Hills	***Sophora prostrata*** *Asplenium flabellifolium* *Polystichum oculatum* *Muehlenbeckia complexa* *Poa cita* *Cytisus scoparius* (exotic)
L2 Mt Cass	***Carmichaelia australis*** ***Discaria toumatou*** *Muehlenbeckia complexa* *Coprosma rhamnoides* *Coprosma propinqua* *Asplenium flabellifolium* *Polystichum neozelandicum* *Pittosporum tenuifolium* *Pseudopanax arboreus*	L6 Little river	***Sophora microphylla*** *Hoheria angustifolia* *Melicytus ramiflorus* *Hedycarya arborea* *Pittosporum eugenioides* *Parsonsia heterophylla* *Muehlenbeckia australis* *Passiflora tetrandra* *Piper excelsum* *Coprosma rotundifolia* *Coprosma robusta* *Solanum aviculare*
L3 Mt Cass	***Sophora microphylla*** *Podocarpus totara* *Cordyline australis* *Melicytus ramiflorus* *Griselinia littoralis* *Muehlenbeckia australis* *Rubus squarrosus* *Pennantia corymbosa* *Coprosma propinqua* *Pseudopanax arboreus* *Pittosporum eugenioides* *Pellaea rotundifolia* *Asplenium appendiculatum* *Asplenium lyalli* *Dryopteris filix-mas* (exotic) *Sonchus oleraceus* (exotic)	L7 Mt Bossu	***Carmichaelia australis*** ***Discaria toumatou*** *Melicytus alpinus* *Coprosma rhamnoides* *Coprosma areolata* *Coprosma propinqua* *Pseudopanax arboreus* *Aciphylla aurea* *Phormium tenax* *Podocarpus totara* *Pteridium esculentum* *Wahlenbergia albomarginata* *Acaena novae-zelandiae* *Kunzea ericoides(känuka)* *Microtis unifolia* *Asplenium appendiculatum*

	Solanum aviculare *Arthropodium cirratum* *Urtica ferox*		*Gaultheria depressa*
L4 Waipara Gorge	***Sophora prostrata*** ***Carmichaelia australis*** *Muehlenbeckia complexa* *Melicytus alpinus* *Echium vulgare* (exotic)		

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